Regulation of shoot development in maize via brassinosteroid signaling

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Regulation of shoot development in maize via brassinosteroid signaling

by

Gokhan Kir

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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CHAPTER 1. GENERAL INTRODUCTION

In contrast to animals, plants do not have the ability to move from one place to another in response to physiological and environmental changes. However, they have skills with which they respond effectively to changes affecting them. Many of these skills come from the chemical compounds they produce, including plant hormones. Some of the roles of these hormones in plant development and growth are: root development, flower development, seed development and germination, stem elongation, cell division, leaf growth, and involvement in biotic and abiotic stresses. Auxins, gibberellins, cytokinins, abscisic acid, and ethylene are known as the “classical five phytohormones” (Kende and Zeevaart, 1997). In addition to these, brassinosteroids (BRs), which are the focus of this dissertation, are considered as the 6th phytohormone and compared to other hormones are a relatively new member of this hormone family.

Brassinosteroids (BRs)

BRs are plant steroid hormones with many roles in plant development, growth, and stress responses. They were first isolated from pollen of Brassica napus, so they were named after this genus (Grove et al., 1979). Recognition of BRs as plant hormones occurred when the first BR-related genes were cloned (Li et al., 1996; Fujioka et al., 1997). DET2 was the first gene cloned in the BR biosynthesis pathway in Arabidopsis and det2 mutants have developmental defects such as reduced plant height, late flowering, dark color leaves, and disrupted male fertility (Chory et al., 1991; Li et al., 1996; Fujioka et al., 1997). det2 mutants could be rescued by exogenous brassinolide and DET2 was found to encode a 5α-reductase which catalyzes 5α-reduction of (24R)-ergost-4-en-3-one to (24R)-5α-ergostan-3-one, (22S, 24R)-22-hydroxyergost-
4-en-3-one to (22S, 24R)-22-hydroxy-5α-ergostan-3-one and (22S)-22-hydroxycholest-4-en-3-one to (22S)-22-hydroxy-5α-cholestan-3-one (Fujioka et al., 1997; Noguchi et al., 1999; Fujioka et al., 2002). With the recognition of BRs as phytohormones, efforts were made to reveal more about their biosynthesis and signaling. The first gene cloned from BR signaling was BRI1, which encodes a receptor kinase located on the plasma membrane and responsible for BR binding and initiation of BR signal transduction (Clouse et al., 1996; Li and Chory, 1997). Subsequent work identified the key players involved in BR signaling in model organisms.

**Brassinosteroid signaling**

In brief, BR signaling occurs as follows: BRs are bound at the cell membrane by BRI1, which is a receptor kinase protein (Li and Chory, 1997; Li et al., 2002; Kinoshita et al., 2005). In the absence of BRs, BRI1 is bound and inhibited by BKI1, which is a negative regulator of the pathway (Wang and Chory, 2006). Binding of BRs at the cell membrane cause BRI1 activation and dissociation of BKI1. Activated BRI1 interacts with another membrane located kinase, BAK1, and this interaction increases BRI1’s activity (Nam and Li, 2002). Activated BRI1 phosphorylates and activates the protein kinases BSK1 and CDG1 (Tang et al., 2008; Kim et al., 2011). Following this, BSU1, a phosphatase protein with kelch-repeat domain, is phosphorylated and activated by BSK1. Activated BSU1 then dephosphorylates BIN2, which is another negative regulator of the pathway (Li et al., 2001; Li and Nam, 2002; Mora-Garcia et al., 2004; Ryu et al., 2010). Dephosphorylation inactivates BIN2 thereby allowing BR transcription factors family, BES1/BZR1, to accumulate in the nucleus and cause BR related gene expression (Wang et al., 2002; Yin et al., 2002). In the absence of BRs, BIN2 is active and phosphorylates BR transcription factors to target them for degradation, thus inhibiting their nuclear accumulation (He et al., 2002; Wang et al., 2002; Yin et al., 2002).
Detailed review of three key players BRI1, BIN2, and BES1, which are the focus of this study, is given below.

**BRI1 (BRASSINOSTEROID INSENSITIVE1), the BR receptor**

*BRI1* (Brassinosteroid Insensitive 1) was identified from a mutant screen where the recessive *bri1* mutant did not respond to exogenously applied brassinolide, a highly active type of BRs (Clouse et al., 1996). Mutant plants were characterized by their reduced stature, dark green colored and thicker leaves, and reduced male fertility (Clouse et al., 1996). While BRI1 expression is high in young growing tissues, it is expressed at low levels in older tissues in Arabidopsis (Friedrichsen et al., 2000).

*BRI1* encodes for a membrane localized LRR (leucine-rich repeat) serine/threonine receptor kinase, involved in BR signaling in Arabidopsis (Clouse et al., 1996; Li and Chory, 1997). It has a right-handed superhelix structure with 25 LRR domains (Hothorn et al., 2011). In addition to being a serine/threonine protein kinase, the ability to be auto-phosphorylated on several tyrosine residues and phosphorylate BKI1 tyrosine residues can classify it as a dual specificity kinase as well (Oh et al., 2009; Jaillais et al., 2011).

BRI1 protein contains an extracellular domain, a transmembrane domain, and a protein kinase domain. The extracellular domain has 25 LRRs and between the 21st and 22nd LRR repeats is a 70 aa long unique domain called the Island Domain (ID), which is important for BR binding (Kinoshita et al., 2005). In addition to the island domain, studies showed that the 22nd LRR repeat, which is right after the ID, is also important for BR binding (Kinoshita et al., 2005). While the extracellular domain is important for BR binding, the kinase domain is essential for intracellular activity of BRI1 and mutations in this domain cause nonfunctional BRI1 protein (Li and Chory, 1997).
Similar to BRI1, BAK1, co-receptor of BRI1, is also a LRR kinase, which has no BL, the most active BRs in Arabidopsis, binding capacity, located on the plasma membrane (Li et al., 2002; Wang et al., 2005). Binding of BL to BRI1 increases BRI1-BAK1 interaction and this interaction on the plasma membrane further increases their activation with the help of trans-phosphorylation of each other (Nam and Li, 2002; Sun et al., 2013). BL binding to BRI1 increases its function to some extent, but in order to be fully functional it requires interaction with BAK1. This happens in the following order; BL bound BRI1 transphosphorylates the kinase domain of BAK1 first and activates it and in turn, activated BAK1 transphosphorylates BRI1 thus increasing its kinase activity and enabling it to function at full capacity (Wang et al., 2008).

There are several mechanisms that turn off BR signaling through BRI1. In the absence of BRs, BRI1 is inhibited by BKI1, which prevents its association with BAK1 and keeps BRI1 activity at low levels. Activated BRI1 phosphorylates BKI1 at S270 and S274 sites as well as on Y211 and triggers its dissociation from plasma membrane into the cytosol (Jaillais et al., 2011; Wang et al., 2011). In addition to BKI1, the C-tail of BRI1 also inhibits its function acting as a autoinhibitory mechanism (Wang et al., 2005; Wang and Chory, 2006). Another mechanism which decreases BR signaling is inhibitory effect of auto-phosphorylation of serine-891 of BRI1 (Oh et al., 2012). Studies also showed that PP2A dephosphorylates BRI1 and causes its degradation to turn off BR signaling (Di Rubbo et al., 2011; Wu et al., 2011).

In addition to being plasma membrane located, BRI1 is also located in endosomes. Studies have shown that BRI1 undergoes constitutive endocytosis, and this process is independent of active BR signaling (Russinova et al., 2004; Geldner et al., 2007). While BRI1 homodimerizes at the plasma membrane, it heterodimerizes with BAK1 in endosomes and BAK1 increases BRI1’s endocytosis (Russinova et al., 2004).
In Arabidopsis, there are three *BRI1-like (BRL)* genes, *BRL1*, *BRL2*, and *BRL3* (Cano-Delgado et al., 2004; Zhou et al., 2004; Ceserani et al., 2009). While BRL1 and BRL3 proteins have the capacity for BR binding, BRL2 does not (Kinoshita et al., 2005). In Arabidopsis, BRI1 has a higher expression level and it is ubiquitously expressed in many tissues, while BRL1 and BRL3 have lower expression levels and are expressed mainly in vascular tissues (Cano-Delgado et al., 2004; Zhou et al., 2004) and the root apex (Cano-Delgado et al., 2004; Fabregas et al., 2013). Overexpression of BRL1 or BRL3 partially rescued a weak *bri1* mutant in Arabidopsis, and a triple mutant *bri1 brl1 brl3* showed a more severe phenotype than a single *bri1* mutant (Cano-Delgado et al., 2004; Zhou et al., 2004). These results suggest that BRL1 and BRL3 are partially redundant with BRI1 in BR signaling of Arabidopsis.

Similar to Arabidopsis, rice contains a BRI1 ortholog and two additional proteins capable of binding BRs, OsBRL1 and OsBRL3, but not OsBRL2. Unlike OsBRI1, OsBRL1 and OsBRL3 are mainly expressed in roots (Nakamura et al., 2006).

**BIN2 (BR-Insensitive-2)**

*BR-Insensitive-2 (BIN2)* was identified as a semidwarf plant from a mutant screen where it was resistant to exogenously applied BRs in Arabidopsis (Li et al., 2001; Li and Nam, 2002). Arabidopsis *bin2* mutants resemble classic BR loss of function mutants with dark green color leaves, short plant height, and male sterility (Li et al., 2001). With careful examination, it was found that *bin2* was a gain-of-function mutant with reduced levels of BR signaling. Several studies found that *bin2/ucu1/dwf12* mutants were alleles of a gene encoding a GSK3-like kinase that functions as a negative regulator of BR signaling (Choe et al., 2002; He et al., 2002; Li and Nam, 2002; Perez-Perez et al., 2002). While overexpression of BIN2 causes plants with reduced BR signaling, reduced expression of BIN2 causes plants with increased BR signaling, which
have a phenotype with wavy and longer petioles in Arabidopsis (Li and Nam, 2002; Yan et al., 2009).

BIN2 is a serine/threonine kinase and a member of Glycogen Synthase Kinase-3 (GSK3) family (Li and Nam, 2002). BIN2 protein has three main parts; an N-terminal domain, a kinase domain, and a C-terminal domain. While the kinase domain of BIN2 is highly conserved with other GSK3s in Arabidopsis, the N- and C-terminal domains show more variability. A conserved TREE (Thr-261-Arg-262-Glu-263-Glu-264) motif, located near the catalytic domain, is thought to be important for inhibition of BIN2 by BR signaling, as mutations in this domain caused gain-of-function mutants, with more stable BIN2, so decreased BR signaling (Choe et al., 2002; Li and Nam, 2002; Perez-Perez et al., 2002; Yan et al., 2009).

In the absence of BRs, BIN2 phosphorylates BR transcription factors BZR1/BES1 (BZR2) and triggers their degradation, and therefore inhibits BR regulated gene expression (He et al., 2002). BIN2 interacts with BZR1 via BZR1’s BIN2 Docking Motif (DM), 12 aa long, close to its C-terminal. Deletion of this motif from BZR1, prevents its phosphorylation by BIN2 and its further degradation (Peng et al., 2010). In addition, BIN2 phosphorylation of BZR1 causes 14-3-3 proteins to bind to pBZR1, leading to its exclusion from the nucleus (Bai et al., 2007). However, in the presence of BRs, BIN2 is inactivated and BR transcription factors accumulate in the nucleus and cause BR regulated gene expression. BIN2 inactivation is performed by BSU1, a phosphatase with a Kelch-repeat domain. When activated by BR signaling, BSU1 dephosphorylates BIN2 on pTyr200 and sets BIN2 for proteasome-mediated degradation (Peng et al., 2008; Kim et al., 2009). Thus, Tyr200 is a conserved residue which is required for normal BIN2 function to inhibit nuclear accumulation of BR transcription factors (Kim et al., 2009).
While there are two members of GSK3 in animals, GSK3 alpha and beta, they are represented with multiple members in plant species (Jonak and Hirt, 2002; Saidi et al., 2012). For example, they are represented with ten members in Arabidopsis and nine in rice (Jonak and Hirt, 2002). GSK3s have a wide range of roles in plant growth and development such as cell elongation, flower development, hormone signaling, and stress tolerance (Piao et al., 1999; He et al., 2002; Claisse et al., 2007; Yan et al., 2009). Phylogenetic analyses divided Arabidopsis GSK3 into four subgroups (Jonak and Hirt, 2002; Saidi et al., 2012). Among these, all “group II” members function redundantly in BR signaling, with BIN2 being predominant (Yan et al., 2009).

Besides being involved in BR signaling by phosphorylating and inhibiting BES1/BZR1, BIN2/GSK3s are also involved in regulating some other growth and development related pathways and biotic and abiotic stresses. BIN2 interacts and inhibits YDA and allows downstream elements to start stomata development in Arabidopsis. This pathway is BR signaling dependent as presence of BRs inhibits BIN2 and stomata development (Kim et al., 2012). Plants that have higher expression of BIN2 have more stomata clustering showing a positive effect of this kinase on stomata development in leaves (Kim et al., 2012). In contrast to these results, another study showed that BRs increase stomata development in Arabidopsis hypocotyls through BIN2 regulated SPEECHLESS (SPCH) transcription factors, which are involved in stomata development. Blocking BIN2 activity increases SPCH stabilization to increase stomata development (Gudesblat et al., 2012). BIN2 also sits at a critical point between BRs and auxin. Vert et al. showed that BIN2 interacts and phosphorylates auxin response factor 2 (ARF2) and decreases its DNA binding activity, which shows a direct relation between BRs and auxin (Vert et al., 2008). A different study shows BIN2’s positive role in auxin as well (Maharjan et al., 2011). There also appears to be cross-talk between BRs and Abscisic acid (ABA) through BIN2.
Zhang et al. presented that ABA was able to inhibit BR signaling in a \textit{bri1} mutant, but not when BIN2 homologs are inhibited, which suggests a possible mechanism where BR inhibition by ABA is regulated through BIN2 kinase or some other elements between BRI1 and BIN2 (Zhang et al., 2009).

GSK3s have other roles in plant development besides being directly or indirectly involved in BR signaling. AtSK11 and AtSK12, two members of clade I, are involved in flower development. Plants with decreased expression of AtSK11 and AtSK12 had flowers with more petals and sepals and disrupted gynoecia. Expression of these proteins in developing WT flowers is consistent with their role in normal flower development in Arabidopsis (Dornelas et al., 2000). Similar to these GSK3s, AtSK32, a group III member, is localized to floral meristems and male and female gametophytes suggesting its role in inflorescence development (Tavares et al., 2002). AtSK32 is also involved in embryo development and controlling cell elongation of floral organs (Tavares et al., 2002; Claisse et al., 2007).

Besides these developmental roles, GSK3 members are also involved in stress responses. In a study where expression level of all Arabidopsis GSK3 were checked, AtSK13, AtSK31, and AtSK42 responded to osmotic pressure and saline treatment (Charrier et al., 2002). In addition to these, a group II member, AtSK22, also responded to NaCL treatments (Piao et al., 1999). In a different study, an alfalfa GSK3 gene, WIG, showed increased expression in response to wounding (Jonak et al., 2000). Another alfalfa GSK3, MsK1, has been shown to be involved in pathogen response signaling. Plants that were overexpressing MsK1 had more susceptibility to pathogen \textit{Pseudomonas syringae} (Wrzaczek et al., 2007). These all show that GSK3 members have many various roles in plant growth and development as well as stress responses.
**BES1 (bri1-EMS-suppressor 1)**

BES1 first was identified from an EMS suppressor screen, where a semi-dominant mutant rescued a weak *bri1* mutant in Arabidopsis (Yin et al., 2002). The *bes1-D* mutant, which was a gain of function mutant, had increased BR response with elongated petioles, curly leaves, and early senescence (Yin et al., 2002). A BES1::GFP construct showed that, BES1 accumulates in the nucleus in response to BL treatment (Yin et al., 2002). BES1 has high similarity with another BR transcription factor, BZR1, and both regulate gene expression in response to BR signaling (Wang et al., 2002).

In the absence of BRs, BES1 is phosphorylated by BIN2 and is targeted for degradation (He et al., 2002). BES1’s activity is inhibited by several mechanisms, including proteasome-mediated degradation, nuclear export and inhibition of DNA-binding activity (He et al., 2002; Gampala et al., 2007; Peng et al., 2008). In the presence of BRs, since BIN2 is inactivated, BES1/BZR1 accumulate in the nucleus and cause BR-related gene expression, leading to BR-regulated growth and development. BES1 and BZR1 both bind to promoter regions of many genes, which are either negatively or positively regulated by BRs. BES1 binds to CANNTG sequences (E-box), which are located on the promoter of genes regulated by BRs (Yin et al., 2005).

In a ChIP-chip study, it was revealed that 953 BR-regulated genes are direct targets of BZR1 in Arabidopsis (Sun et al., 2010). In a different study on BES1, 1609 BES1 targets were found in Arabidopsis, among which 404 genes are regulated by BRs either positively or negatively (Yu et al., 2011). BES1 recruits cofactors that are involved in BR regulated gene expression. BIM1, IWS1, MYB30, and MYBL2 are some elements that BES1 interacts with in BR signaling (Yin et al., 2005; Li et al., 2009; Li et al., 2010; Ye et al., 2012). BIM1 was
identified from a yeast two-hybrid screen as a protein interacting with C-domain of BES1 (Yin et al., 2005). BES1 and BIM1 interact with each other and together bind to E-box sequences in numerous BR-induced genes (Yin et al., 2005). BES1 binds AtIWS1 and engages it in transcription elongation of BR induced genes. Loss of function atiws1 mutants are dwarf and have decreased BR response (Li et al., 2010). AtMYB30 was identified from microarray and ChIP experiments as a transcription factor, which interacts with BES1. Null AtMYB30 mutants had changed BR response and increased the phenotype of a weak bri1 mutant, suggesting AtMYB30’s positive role in BR signaling. It was suggested that BES1 and AtMYB30 work together to increase BR regulated gene expression (Li et al., 2009). In contrast to AtMYB30, it was found that BES1 recruits MYBL2 to reduce expression of BR-repressed genes (Ye et al., 2012). In addition to its interaction with BES1, MYBL2 is phosphorylated by BIN2, which makes it more stable, a negative regulator kinase involved in BR signaling (Ye et al., 2012). Studies showed that BR signaling also induces histone modifications to regulate downstream gene expression (Yu et al., 2008; Lu et al., 2011; Sui et al., 2012).

Efforts on these key players revealed much about their functions in BR signaling in model organisms. Yet, their understanding in maize, which is an important crop, remains very poor. To address this problem, we took a transgenic approach to explore these players’ functions in BR signaling in maize and BR signaling’s role in maize development. The outcome of this research will be useful to manipulate this important crop to answer the world’s growing food demand.

**Dissertation Organization**

This dissertation includes the general introduction (Chapter 1), two chapters in the format of journal manuscripts (Chapter 2 and 3) and a general conclusion (Chapter 4).
In Chapter 2, we analyzed the effects of BRI1/BRL family suppression in maize growth and development by generation of bri1-RNAi transgenic lines. My contribution included generation of the RNAi construct, phylogenetic analysis of BRI1/BRL family in maize, molecular and phenotypic characterization of the bri1-RNAi lines, recovery and confirming BES1-YFP marker is BR inducible, microscopy work, and writing the manuscript. This manuscript has been submitted for publication and is currently under review at the time of thesis submission.

In Chapter 3, effects of suppression of GSK3/BIN2 kinases via RNAi on maize growth and development were analyzed. My contribution included phylogenetic analyses of GSK3/BIN2 family in maize, molecular and phenotypic characterization of bin2-RNAi lines, generating and analyzing bri1-RNAi : bin2-RNAi double transgenic lines, microscopy work, and writing the manuscript.

References


CHAPTER 2. RNAi KNOCKDOWN OF BRI1 IN MAIZE (ZEA MAYS) REVEALS NOVEL FUNCTIONS FOR BRASSINOSTEROID SIGNALING IN CONTROLLING PLANT ARCHITECTURE

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Abstract

Brassinosteroids (BRs) are plant hormones involved in various growth and developmental processes. The BR signaling system is well established in Arabidopsis (Arabidopsis thaliana) and rice (Oryza sativa) but poorly understood in maize (Zea mays). BRI1 is a BR receptor and database searches and additional genomic sequencing identified 5 maize homologs including duplicate copies of BRI1 itself. RNA interference (RNAi) using the extracellular coding region of a maize zmbr1 cDNA knocked down expression of all 5 homologs. Decreased response to exogenously applied brassinolide (BL), and altered BR marker
gene expression demonstrate that zmbr1-RNAi transgenic lines have compromised BR signaling. zmbr1-RNAi plants showed dwarf stature due to shortened internodes, with upper internodes most strongly affected. Leaves of zmbr1-RNAi plants are dark green, upright and twisted, with decreased auricle formation. Kinematic analysis showed that decreased cell division and cell elongation both contributed to the shortened leaves. A BES1-YFP transgenic line was developed that showed BR inducible BES1-YFP accumulation in the nucleus, which was decreased in zmbr1-RNAi. Expression of the BES1-YFP reporter was strong in the auricle region of developing leaves, suggesting that localized BR signaling is involved in promoting auricle development, consistent with the zmbr1-RNAi phenotype. The blade/sheath boundary disruption, shorter ligule and disrupted auricle morphology of RNAi lines resembles KNOX mutants, consistent with a mechanistic connection between KNOX genes and BR signaling.

**Introduction**

Brassinosteroids (BRs) are ubiquitous plant hormones that promote plant growth by regulating cell elongation and division (Clouse, 1996; Clouse et al., 1996). BRs have other diverse roles including enhancing tracheary element differentiation, stimulating ATPase activity, controlling microtubule orientation, controlling flowering time, fertility, and leaf development (Chory et al., 1991; Iwasaki and Shibaoka, 1991; Clouse et al., 1996; Schumacher et al., 1999; Catterou et al., 2001; Oh et al., 2011). BRs also function in tolerance to both biotic and abiotic stresses such as extreme temperatures, drought, and pathogens (Krishna, 2003).

Deficiencies in BR biosynthesis or signaling produce characteristic dwarf plant phenotypes (Clouse et al., 1996; Szekeres et al., 1996; Szekeres et al., 1996; Fujioka et al., 1997; Fujioka et al., 1997). Plant height is an important agricultural trait as seen in the Green Revolution, where semi-dwarf mutants contributed to increased yields in small grain crops (Salas
BR deficient dwarf rice produced increased grain and biomass yields because the erect leaf habit allowed higher planting densities under field conditions (Sakamoto et al., 2006). In fact, Green Revolution ‘uzu’ barley (*Hordeum vulgare*) is based on a mutation of the *uzu1* gene, which encodes a homolog of BRASSINOSTEROID INSENSITIVE1 (BRI1), a BR receptor (Chono et al., 2003).

Genes functioning in BR pathways have been identified by analysis of dwarf mutants in several species, including Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*). Arabidopsis *bri1* mutants are shortened, have reduced apical dominance and are male-sterile (Clouse et al., 1996). *BRI1* encodes a leucine-rich repeat receptor-like kinase (LRR RLK) that is located in the plasma membrane and contains an extracellular domain responsible for BR binding, a transmembrane sequence, and a cytoplasmic protein kinase domain (Li and Chory, 1997; Vert et al., 2005; Belkhadir and Chory, 2006). The Island Domain (ID) and subsequent LRR22 domain are critical for BR binding (Kinoshita et al., 2005; Hothorn et al., 2011; She et al., 2011). Phosphorylation of conserved residues Ser-1044 and Thr-1049 in the kinase activation loop activates the BRI1 kinase (Wang et al., 2005), while dephosphorylation of BRI1 by protein phosphatase 2A (PP2A) inhibits its function (Wu et al., 2011).

BRI1 is partially redundant in BR signaling with related *BRI1-LIKE RECEPTOR KINASE (BRL)* paralogs, both in Arabidopsis and rice. In Arabidopsis, even though null alleles of *brl1* or *brl3* did not show obvious phenotypic defects in shoots, they enhanced the developmental defects of a weak *bri1*-5 mutant. In contrast to ubiquitously expressed *BRI1*, *BRL1*, 2 and 3 are tissue specific, mostly expressed in vascular tissues, while *BRL1* and 3 are also expressed in root apices (Cano-Delgado et al., 2004; Zhou et al., 2004; Fabregas et al., 2013). Both BRL1 and BRL3 can bind BL (Cano-Delgado et al., 2004). In rice, *OsBRI1* is
similar to the Arabidopsis BRI1 gene and phenotypes of OsBRI1 rice mutants include dwarf plants with shortened internodes, erect leaves that are twisted and dark green, and photomorphogenesis in the dark (Yamamuro et al., 2000). There are three BR receptors in rice as well, and while OsBRI1 is universally expressed in all organs, OsBRL1 and OsBRL3 are expressed mostly in roots (Nakamura et al., 2006).

To date, two mutant genes of the BR biosynthetic pathway have been reported in maize (Zea mays). A classic dwarf mutant, nana plant1 (na1), has a mutation in a DET2 homologous gene, which encodes a 5α-reductase enzyme in the BR biosynthesis pathway (Hartwig et al., 2011), while the brd1 gene encodes brC-6 oxidase (Makarevitch et al., 2012). The maize BR deficient mutants have shortened internodes, twisted, dark green, erect leaves and feminized male flowers (Hartwig et al., 2011; Makarevitch et al., 2012). However, no gene functions in BR signaling have yet been reported in maize. Understanding BR signaling in maize might help improve this important crop for production of biofuels, biomass and grain yield. Here, we took a transgenic RNAi approach to generate maize plants partially deficient for BRI1. These knockdown lines demonstrate that BRI1 functions are generally conserved in maize compared to other plant species, but they also exhibit unique phenotypes suggesting either that maize possesses novel BR-regulated developmental processes, or that aspects of maize morphology reveal processes not evident in other plants.

Results

BRI1 homologs in maize

BRI1 belongs to a family of LRR receptor-like kinases and at least 2 BRI1-like genes are involved in BR signaling in Arabidopsis (Cano-Delgado et al., 2004; Zhou et al., 2004). Maize BRI1 homologs were identified by using the Arabidopsis and rice BRI1 amino acid sequences to
search the maize genome. A phylogenetic analysis was carried out to identify relationships among maize BRI1 homologs. Protein products were aligned (Supplemental Fig. S1) and a phylogenetic tree obtained by the neighbor-joining method (Fig. 1). Five putative maize genes encode proteins belonging to the BRI1 family (Table 1). Figure 1B shows that two maize gene products are most closely related to Arabidopsis and rice BRI1 while three additional genes encode proteins similar to BRL1, BRL2 and BRL3. The top hit, herein designated zmbr1a, corresponds to GRMZM2G048294 on chromosome 8. The ZmBRI1a protein product shows 54 % amino acid identity and 69 % similarity to Arabidopsis BRI1 (AtBRI1) and 79 % amino acid identity and 88 % similarity to rice BRI1 (OsBRI1). A second gene, GRMZM2G449830, located on chromosome 5 contained only a partial sequence in the genome assembly (B73 v.2) but was found through cloning and sequencing to contain a complete gene, designated zmbrilb (Genbank accession number KP099562). The predicted ZmBRI1b protein shows 93 % amino acid identity and 95 % similarity to maize ZmBRI1a, 79 % amino acid identity and 88% similarity with OsBRI1, and 54 % amino acid identity and 69 % similarity with AtBRI1. The island domain or insertion domain between LRRs 21 and 22 is a distinguishing characteristic of the BRI1 family of receptor kinases and the site of BR binding in BRI1 (Kinoshita et al., 2005; Hothorn et al., 2011; She et al., 2011). All five maize BRI1/BRL homologs contained a conserved island domain and LRR 22 sequences involved in BR binding (Fig. 1C).

The expression patterns of these five BRI1/BRL homologs were examined by querying publicly available gene expression datasets. According to the Maize Gene Expression Atlas (Sekhon et al., 2011), the zmbr1a and zmbrilb transcripts are widely expressed in most tissues sampled, with zmbr1a showing somewhat higher expression levels and a more ubiquitous pattern than zmbrilb (Supplemental Fig. S2). Notably, both are expressed in shoot apices,
developing leaves, internodes and roots. The *zmbrl* genes all show generally lower steady state transcript levels than the *zmbrl1* genes except in a couple instances such as roots and germinating seeds where *zmbrl* transcripts are present at higher levels than *zmbrl1b*. The *zmbrl1* and *zmbrl3* transcripts are more highly expressed than *zmbrl2* except in roots where all 3 genes are expressed at comparable levels. In most tissues, the *zmbrl2* transcript was below reliable detection levels.

Expression along the developmental gradient of a growing seedling leaf was also examined (Wang et al., 2014). As shown in Supplemental Fig. S3, *zmbrila* and *zmbrilb* expression was high at the base of the leaf in the zones of active cell division and cell elongation, gradually declining as cells differentiate and mature. Expression of the *zmbrl* genes was generally very low except for a brief increase in *zmbrl1* transcript levels near the region of the leaf where tissues change from acting as carbon sinks and become photosynthetically active as source tissue.

**zmbrl1-RNAi lines show compromised BR signaling**

To generate loss-of-function RNAi suppression of maize BRI1-related genes, a 498bp fragment encoding LRRs 6 to 12 of the extracellular domain in the protein product was cloned in forward and reverse orientations into a pMCG1005 vector, controlled by the maize ubiquitin1 promoter and transformed into maize. Among 17 independent *bri1*-RNAi T0 transgenic events, 13 showed clear dwarf phenotypes similar to those reported in rice *d6l* or maize *na1* seedlings (Yamamuro et al., 2000; Hartwig et al., 2011). Two events producing strong and intermediate phenotypes were selected and backcrossed three or more generations to B73 inbreds.

Because BRI1 belongs to a gene family, it is likely that multiple members participate in BR signaling, perhaps redundantly, and possible that multiple members were targeted by the RNAi construct. To determine which genes in the maize genome were likely to be targeted by the *bri1*-RNAi construct, nucleotide database searches were conducted using the *zmbrila* cDNA
fragment contained in the RNAi construct. The results of this search were consistent with the phylogeny shown in Fig.1B. The two top hits included the original \textit{zmbrila} and \textit{zmbrilb} with 93\% nucleotide identity. The \textit{zmbril} homologs showed somewhat lower similarity ranging from 43.9\% to 50.4\% nucleotide identity, although localized stretches of nucleotides showed higher levels of homology (alignments shown in Supplemental Fig. S4). The expression of these 5 genes was examined using RNA obtained from young developing shoot tissue, which included meristems. Both \textit{zmbril}-RNAi lines showed decreased expression of all five BRII homologs in leaves and internode tissues compared to non-transgenic siblings (Fig.1D).

Expression of BR marker genes \textit{brd1} and \textit{cpd}, encoding enzymes involved in BR biosynthesis, was examined by qRT-PCR. These genes are negatively feedback regulated by BR signaling in Arabidopsis and rice, causing increased expression in mutants with decreased BR signaling (Mathur et al., 1998; Hong et al., 2002; Bai et al., 2007). As seen in Fig. 2, both leaf and stalk internode tissues have increased expression of \textit{cpd} and \textit{brd1}, consistent with decreased BR signaling activity in strong \textit{bri1}-RNAi lines compared to non-transgenic siblings.

To further analyze BR signaling, we generated a BES1-YFP reporter for BR activity. BES1 is a transcription factor that is post-translationally stabilized and translocated into the nucleus to regulate gene expression in response to BR signaling (Yin et al., 2002). As such, BR signaling is expected to increase nuclear fluorescence due to BES1-YPF accumulation (Ryu et al., 2010). The maize genome was searched for BES1 homologs and GRMZM2G102514 was clearly the top candidate. The predicted protein encoded by this gene model contains 313 amino acids and shows 50\% overall identity, 62\% similarity to Arabidopsis BES1 and 78\% identity and 82\% similarity to OsBZR1. To create a stable BES1-YFP maize line, the YFP coding sequence was translationally fused to the 3’ end of the maize BES1 coding region within the native
genomic context of the gene, including 5’ and 3’ regions as well as introns (Mohanty et al., 2009). As expected, nuclear fluorescence in leaf sheath cells is BR inducible in transgenic BES1-YFP lines, indicating the fusion protein responds to BRs and can serve as a BR reporter (Fig. 2C).

Since BES1-YFP responds to BRs, a decrease in BR signaling should cause less BES1-YFP accumulation in the nucleus. To test the hypothesis that zmbr1-RNAi disrupts BR signaling, we examined several tissues in zmbr1-RNAi; BES1-YFP lines. As seen in Fig. 2C, there is decreased nuclear fluorescence in zmbr1-RNAi leaf sheath cells, compared to the WT cells, consistent with decreased levels of BR signaling and BES1-YFP accumulation. To see if these transgenic cells were able to respond to BR, we exposed both transgenic and non-transgenic leaf sheath cells to 1μM BL. Fig. 2C shows that WT cells are more responsive to BL than RNAi cells confirming that BR signaling is impaired in zmbr1-RNAi lines.

To further confirm disrupted BR signaling in br1-RNAi lines, we performed a BR root growth inhibition assay, a commonly used bioassay for BR sensitivity (Clouse, 1996; Chono et al., 2003; Müssig et al., 2003; Kim et al., 2007; Wang et al., 2007; Hartwig et al., 2012). Germinated seeds were treated with ddH2O (mock), 20 nM BL, or 100 nM BL. After 10 days, WT plants showed a two-fold greater inhibition of root growth compared to br1-RNAi seedlings, indicating decreased BL sensitivity in transgenic seedlings (Fig. 2B and Supplemental Fig. S5).

**zmbr1-RNAi alters maize plant architecture**

In both mild and strong zmbr1-RNAi lines, plant height is decreased compared to non-transgenic siblings (Fig.3 and Table 2). Internodes were analyzed to determine the basis of the shorter plant height. While the number of internodes was not significantly changed (Table 2),
Internode length was dramatically shorter in \textit{zmbril}-RNAi lines than their non-transgenic siblings. In the milder lines, internodes are shortened by similar proportions throughout the plant (Supplemental Fig.S6), while in strong lines, internodes between the ear and tassel nodes were most dramatically affected (Fig.3, B-D). Internode epidermal cell lengths were shortened in \textit{zmbril}-RNAi lines compared to their non-transgenic counterparts, suggesting decreased cell elongation is a major contributing factor to the decreased internode length (Fig.3, E-G).

Leaf blades and sheaths were both shorter in RNAi lines (Fig. 4, Table 2). Adult leaves in \textit{zmbril}-RNAi transgenic lines were dark green, upright, thickened, and had a wrinkled surface, compared to non-transgenic lines (Fig.3A, Fig. 4C and Supplemental Fig. S7). These phenotypes were more apparent in adult leaves than in seedlings, and stronger in field-grown plants than in the greenhouse. Like internode epidermal cells, leaf epidermal cells of \textit{zmbril}-RNAi lines were also shortened compared to non-transgenic siblings (Fig. 4D,E). Interestingly, cells show increased depth (Fig 4B,C), even though their lengths were decreased, indicating a role for BR signaling in regulating directional cell growth. In the lines with the strongest phenotype, upper leaves strongly enclasped the tassel, necessitating manual unfurling to allow pollen release (Fig. 3A). Transgenic lines with mild phenotypes showed a delay in flowering (Table 2) but the enclosed tassels of strong \textit{zmbril}-RNAi lines prevented accurate determination of anthesis onset.

**BRI1 regulates cell division and cell expansion in maize leaf growth**

To understand the cellular basis of the \textit{zmbril}-RNAi shortened leaf phenotype, we performed a kinematic growth analysis on the fourth seedling leaf (Nelissen et al., 2013). This analysis quantifies growth parameters including cell size, cell division rates, cell expansion rates, and the extents of the cell division and cell expansion zones on the leaf, thus allowing the number of cells undergoing each process to be calculated. After backcrossing to B73, seedling
leaves did not display the dramatic corkscrew appearance or other morphological disturbances displayed by adult leaves. Nonetheless, the final length of seedling leaf 4 was decreased (Table 3). Following the growth of the fourth leaf over time showed that the average growth rate or leaf elongation rate (LER) during steady state growth was significantly reduced for the zmbri1-RNAi (Fig. 4F). At the cellular level, both cell expansion, as measured by the final cell size, and cell production were reduced by 7.6% and 7.9%, respectively (Table 3). The kinematic analysis further showed that zmbri1-RNAi impacted cell division by affecting both the number of dividing cells and the rate at which they divide (Table 3). The WT siblings have 26% more dividing cells, while the bri1-RNAi leaves showed cell division rate increased by 15.5% (cell cycle duration decreased by 16.03%), and the size of the zone of cell division and the number of dividing cells were decreased by 38.12% and 26.02%, respectively. In conclusion, our data demonstrate that the reduced leaf size of the zmbri1-RNAi lines is due to decreased cell number and cell size indicating that BRI1 signaling regulates both cell division and cell expansion.

zmbri1-RNAi plants show leaf auricle phenotypes that resemble KNOX mutants

There was also a notable effect on auricle development (Fig. 5) as reported for the maize brd1 mutant (Makarevitch et al., 2012). Indeed, auricles are largely missing in zmbri1-RNAi plants (Fig. 5, B,C). The auricle regions of zmbri1-RNAi leaves are thickened compared to non-transgenic, even more so than in the lamina (Supplementary Fig. S7). In grass leaves, the auricle is a hinge-like structure located between the blade and sheath, which allows the blade to attain its normal angle (Moreno et al., 1997). In rice, BR mutants cause a decreased leaf angle and erect leaf habit compared to WT (Yamamuro et al., 2000; Morinaka et al., 2006), whereas BR gain-of-function mutants show increased lamina joint bending (Li et al., 2009). The altered auricle morphology of zmbri1-RNAi plants led us to hypothesize that BR signaling might have a
specific function in maize auricle development. Consistent with these morphological changes, a striking accumulation of the BES1-YFP marker for BR activity was observed in nuclei of cells in the ligule-auricle region of developing maize leaves (Fig. 5D,E). The band of increased BES1-YFP expression is sharply delineated at the distal end, consistent with a proposed boundary function separating the sheath and blade (Tsuda et al., 2014). The width of the auricle band was narrower and nuclear BES1-YFP fluorescence intensity was significantly lower in the auricle region of zmbr1-RNAi leaves than in WT (Fig 5D,E, Supplemental Table S1). The leaf phenotype of the bri1-RNAi lines bears striking similarity to Knotted1-like homeobox (KNOX) mutants, particularly Rough Sheath1-O (Fig. 6) (Becraft and Freeling, 1994; Schneeberger et al., 1995). Consistent with these phenotypes, the rice KNOX gene OsH1 was recently shown to promote expression of BR catabolic genes (Tsuda et al., 2014). Taken together, these results indicated that BRs function in auricle development to regulate leaf angles.

Discussion

While BR functions are well studied in Arabidopsis and rice (Clouse, 1996; Krishna, 2003; Clouse, 2011), this is the first report on BR signaling components in maize. We took a transgenic approach by targeting BRI1, the BR receptor, with RNAi. The zmbr1-RNAi lines show consistent phenotypes after more than four generations of backcrosses to B73 or other inbred lines. As expected, knockdown of ZmBRI1 caused decreased plant height (Fig.3A and Table 2), consistent with other species (Li and Chory, 1997; Yamamuro et al., 2000). This dwarf phenotype results from reduced internode length due in large part to decreased cell elongation (Fig.3, D-G), consistent with BR control of cell expansion (Friedrichsen and Chory, 2001; Hartwig et al., 2011). Given the results in leaves, it is likely that decreased cell division also
contributes to the shortened internodes. Interestingly, there were differential effects on specific internodes with those above the ear node most strongly affected.

A novel function for BRs in maize sex determination was reported for BR-deficient *nal* mutants (Hartwig et al., 2011). These dwarves exhibit a tasselseed phenotype, where female floral structures fail to abort in male inflorescences. Fertilization of these perfect flowers results in kernels forming on tassels. Tasselseed phenotypes were never observed on our *zmbril*-RNAi lines. It remains to be determined whether this is due to insufficient knockdown of BRI1 function, or due to unknown activities of biosynthetic products of the 5α-reductase enzyme encoded by *nal*.

Leaves of *zmbril*-RNAi plants were short, thick, upright, twisted, and dark green in color. Even though the phenotypes of *bril*-RNAi leaves become stronger in adult plants, a kinematic growth analysis showed seedling leaf #4 was impacted and revealed important information about functions of BR signaling in maize leaf development. Most cellular growth parameters were affected including both cell division and elongation, similar to Arabidopsis (Chory et al., 1991; Szekeres et al., 1996; Azpiroz et al., 1998; Oh et al., 2011; Zhiponova et al., 2013). Decreased cell length indicated that *zmbril*-RNAi inhibited cell elongation. While cell length was decreased, cell diameter was increased in the dorsiventral axis contributing to the thickened blade phenotype and suggesting the effect of BRs on cell expansion is directional (Fig. 4B,C). Kinematic analysis showed that leaf elongation rate was significantly reduced (Fig. 4F). The extent of the cell division zone and number of dividing cells were decreased. Cell division rate and cell cycle duration were also impacted, albeit these parameters were not statistically significant (Table 3).
Several plant hormones affect both cell division and cell expansion (Takatsuka and Umeda, 2014). For example, GA can promote both cell division and expansion (Gonzalez et al., 2010; Gonzalez et al., 2010; Band and Bennett, 2013) and was shown to function at the transition between these processes (Nelissen et al., 2012). In Arabidopsis leaves, BR has also been shown to impact both cell number and cell size (Gonzalez et al., 2010; Zhiponova et al., 2013). In addition, it was shown in the maize leaf that BR levels are high at the base of the leaf, where the actual growth processes of cell division and cell expansion take place (Nelissen et al., 2012). The high expression levels of \( \text{zmbril}a \) and \( \text{zmbril}b \), but not the other \( brl \) genes, suggests the BRI1 receptors encoded by these two genes are of primary importance for regulating leaf growth. The concomitant high BR hormone levels, high expression levels of \( \text{zmbril}a \) and \( \text{zmbril}b \) (Wang et al., 2014) and the observed phenotypes of the \( \text{zmbril} \)-RNAi lines reported here suggest the BR/BRI signaling cascade has a focus of action in the growth zones at the leaf base to regulate leaf size.

A striking phenotype of BR mutants in rice and barley is the upright leaf habit (Yamamuro et al., 2000; Chono et al., 2003), which is an important agricultural trait because upright leaves increase sunlight penetration into a crop canopy (Sinclair and Sheehy, 1999). Breeding improvements to corn yields have arisen not from increases in individual plant yields, but primarily from increased tolerance to higher cropping densities, enabled in large part by changes in shoot architecture (Duvick et al., 2004; Duvick, 2005). Upright leaf habit is a key component of the maize plant ideotype (Mock and Pearce, 1975) and has been correlated with increased yields at high planting densities (Pendleton et al., 1968; Pepper et al., 1977; Lambert and Johnson, 1978). Indeed, a BR-deficient rice \( d4 \) mutant showed increased grain yield due to improved tolerance to higher planting density (Sakamoto et al., 2006). The auricle is a hinge-like
structure at the blade-sheath junction that determines leaf angle and application of BR to the leaf joint in rice is known to increase leaf angle by promoting auricle expansion (Tsuda et al., 2014).

Our results demonstrate a key role for BR signaling in auricle development. The zmbril-RNAi plants showed disrupted auricle morphology and the BES1-YFP reporter suggests a localized concentration of BR signaling activity in the developing auricle (Fig. 5). The disrupted auricle phenotype was highly reminiscent of dominant KNOX (Knotted1-like homeobox) mutants such as Rs1-O (Fig.6) (Becraft and Freeling, 1994; Schneeberger et al., 1995). A disrupted ligule was reported in maize brd1 mutants but the KNOX similarity was not noted (Makarevitch et al., 2012). KNOX genes are important in shoot apical meristems for cells to maintain their pluripotent identities (Hay and Tsiantis, 2009). Downregulation of KNOX genes is required for leaf initiation, therefore expression of these genes is normally repressed in leaf primordia (Hay and Tsiantis, 2010). Altered cell fate and disrupted organ shape are two basic consequences when KNOX genes are ectopically expressed in leaves (Hake et al., 2004). One of the abnormalities caused by mutations that de-repress KNOX genes during leaf development is proximalization, a transformation of blade identity to sheath (Schneeberger et al., 1998; Foster et al., 1999). This phenotype is seen in several maize KNOX gene mutants and is most prevalent in the auricle/ligule region (Schneeberger et al., 1998; Foster et al., 1999).

The phenotypic resemblance between zmbril-RNAi plants and KNOX mutants suggests a mechanistic relation between BRs and KNOX genes in auricle and/or leaf development, and is consistent with recent reports of a regulatory connection between BR and KNOX genes in shoot apical meristems (Tsuda et al., 2014). It was recently reported that the KN1 and OSH1 proteins bind several BR metabolic genes in maize and rice, respectively, and that OSH1 promotes expression of BAS1 homologs (Bolduc et al., 2012; Tsuda et al., 2014). BAS1 encodes a
cytochrome p450 enzyme that catabolizes BRs in Arabidopsis (Tanaka et al., 2005; Turk et al., 2005). Overexpression of OSH1 caused decreased sensitivity of rice auricle tissue to BR application suggesting that KNOX mutants act at least in part by modulating BR levels.

Arabidopsis and rice each contain one gene, BRI1, encoding the major BR receptor, as well as three BRI1-LIKE (BRL) genes, at least two of which contribute to BR signaling (Cano-Delgado et al., 2004; Zhou et al., 2004; Kim and Wang, 2010). Maize contains two close BRI1 homologs, and three BRLs. One of the BRI1 homologs was not complete in the B73 v2 genome assembly, but was found to be intact and expressed. The phylogeny indicates that BRI1 duplicated specifically in maize, while the BRL1, BRL3 pair resulted from independent duplications, one in the Arabidopsis lineage and another in the cereal lineage prior to the divergence of maize and rice. Given the high level of sequence similarity and the similar expression patterns between the maize bri1 homologs, it is highly likely they function redundantly. The RNAi lines reported here were designed using the extracellular domain and decrease expression of all five family members. The relatively low level of nucleotide similarity between the zmbril fragment used for RNAi and the brl genes makes it questionable whether they are direct targets of suppression, or if decreased expression could be an indirect consequence of zmbril suppression. A topic for future studies will be sorting out the various contributions of individual receptor genes to BR signaling and different phenotypic effects in maize.

In summary, we show that BR signaling affects plant height, cell expansion, leaf morphology and auricle development in maize. Similarities between bri1-RNAi plants and KNOX mutants support the relation between BR signaling and KNOX genes. Future studies should help reveal mechanisms of the interactions between these two pathways in leaf
development and the knowledge can be used to help optimize maize architecture for crop production.

**Materials and Methods**

**Sequence analysis**

Sequences used were obtained using BLAST to search maize sequences in NCBI databases including non-redundant protein sequences (nr), Nucleotide collection (nr/nt), Reference RNA sequences (refseq_rna), and High throughput genomic sequences (HTGS) (Altschul et al., 1990). With full-length Arabidopsis BRI1 amino acid sequences, 479 blast hits were returned with scores ranging from 276 to 1105. Rice BRI1 amino acid sequences generated 331 blast hits with scores ranging from 284 to 1699. All alignments were generated with ClustalW and phylogenetic trees were obtained using default parameters of Mega5.1 NJ (Neighbor-joining) method with Poisson model (Larkin et al., 2007; Tamura et al., 2011).

**zm bri1b cloning**

The GRMZM2G449830 gene model, corresponding to the *bri1b* gene, contained 903 bp of transcribed sequence in the genome assembly (B73.v2). The portion of *bri1b* contained in this gene model corresponded to a protein with nine LRR domains and no kinase domain. A combination of PCR cloning and database searching was used to identify the remaining sequence of the *bri1b* homolog. All primers and sequences used for cloning *bri1b* are listed in Supplemental Table S2. Since there was high similarity among the other four BRI1 homologs, initial attempts to identify the 3’ region of *bri1b* were made via PCR with primers based on conserved domains of these related genes. The template consisted of cDNA prepared from B73 seedling leaves using Invitrogen SuperScript® III Reverse Transcriptase kit. Using the Kinase5-R primer, located on kinase domains of other BRI1 homologs, as the reverse primer with a *bri1b* specific forward primer, an additional 1393 bp sequence of *bri1b* homolog was found. By
designing more primers (chr5-R1, R2, R3, R4) based on 3’ sequences of *brila*, another 476 bp of this gene was identified. From this newly identified sequence 55 nucleotides (sequence #1, Supplemental Table S2) were used to search *Zea mays* in NCBI dbEST, and identified three ESTs (gi|32834046, gi|14243881, gi|211181084) that encompassed the remaining 3’ coding region and UTR of *bri1b*. No specific ESTs for the 5’ end of *bri1b* could be found in available databases. With the conserved domain approach for N-terminal of *bri1b*, another additional 196 bp sequence was found by PCR amplification using forward primers (chr5W-F7 and chr5W-F8) based on the 1st LRR domain of *brila*, in combination with the *bri1b* specific qbri1-5R reverse primer. From this part, a 115 bp sequence (sequence #2, Supplemental Table S2) specific to *bri1b* was used for database searches and identified a dbGSS record (gi|34267396) that included the start codon and 5’-UTR. To confirm that all these various fragments originated from a single gene, the entire *bri1b* cDNA was amplified with FideliTaq PCR Master Mix (2X) (affymetrix) proofreading enzyme from B73 seedling leaf cDNA via primers located on 5’UTR (Chr5-N1) and 3’UTR (Chr5-UR). This near full length cDNA was cloned into a TA vector, sequenced at Iowa State University DNA Facility and the sequence deposited in Genbank (accession KP099562).

**Production of transgenic lines**

Because bioinformatic analyses indicated that GRMZM2G048294 (*brila*) encodes a likely maize ortholog of BRII1, a full-length cDNA[(ZM_BFb-Clone) *Zea mays* null cv. B73 0034N07] was obtained from the Arizona Genomics Institute. The *bri1*-RNAi construct was generated by amplifying the region of the cDNA coding for the extracellular domain (bases 611 to 1108 beginning from the start codon). Appropriate restriction sites were added to the primers TGGACCTCTCCGGGAACAAGAT and TGGTGCGATTTGGAGATTGAC and the
fragment was cloned into the pMCG1005 RNAi vector. AvrII and AscI enzymes were used to put the BRI1 fragment in the reverse orientation between the Adhl intron and rice Waxy-a intron, while XmaI and SpeI enzymes were used to put the BRI1 fragment in forward orientation between OCS 3’ and the rice Waxy-a intron. Transgenic lines were identified by glufosinate resistance and expected BR-like mutant seedling phenotype.

**BES1-YFP construction**

The citrine YFP tagged construct, BES1-YFP, was made by fusing the YFP on the C-terminus of BES1 using the MultiSite Gateway method (Invitrogen). Briefly, the native 3139 bp promoter and genomic coding sequence of bes1 was amplified with primers BES1_3GWp1, GGGGACAAGTTTGTACAAAAAAGCAGGCTgcattgatgctatcggagat and BES1_3GWp4, GGGGACAACTTTGTATAGAAAAAGTTGGGGTGcttggegcgcgcgca and the 1300 bp native terminator of bes1 was amplified with primers BES1_3GWp3, GGGGACAACCTTTGTATAATAAGTTTAGtggaagcagcgatcggagat and BES1_3GWp2, GGGGACCACTTTGTACAAAGCTGGGTAcgttctgccgtcttcccgtctc and they were cloned into MultiSite Gateway donor vectors, respectively. The resulting two entry vectors were then recombined into the binary vector pAM1006 (a derivative of pTF101.1 carrying the Gateway Cassette) with the entry vector containing YFP yielding BES1-YFP. BES1-YFP was introduced into Agrobacterium tumefaciens EHA101 and transformed into maize HiII at Iowa State University Plant Transformation Facility. Transgenic lines were identified by glufosinate resistance and evaluated by YFP fluorescence for BES1-YFP expression.

**Semi-quantitative and Quantitative PCR**

Leaf tissues were frozen immediately in liquid nitrogen, ground and kept in -80 °C until RNA purification using a Qiagen RNeasy Mini Kit according to the manufacturer’s protocol.
RNA concentrations were measured by a NanoDrop ND-1000 Spectrophotometer, samples DNase treated (Promega RQ1 RNase-free DNase) and 1-2 μg RNA used per experiment. 1 μl DNase and 1.2 μl RQ1 buffer was used and volumes adjusted to 12 μl with nuclease free water. Samples were incubated at 37°C for 30 minute, after which 1 μl RQ1 DNase stop solution was added and samples kept at 65°C for 10 minutes. 11 μl of this volume was used directly for RT-PCR reactions. RT-PCR was performed with Invitrogen SuperScriptTM III Reverse Transcriptase using the manufacturer’s procedure. From the 20μl cDNA reaction, 1-2 μl was used for further PCR reactions. QRT-PCR was done using iQ SYBR Green Supermix (BioRad) and gene specific primers (Supplemental Table S2) on a Stratagene MX4000 instrument located at ISU DNA Facility

**Phenotypic analyses**

Each transgenic event was backcrossed a minimum of 3 times into a B73 inbred genetic background. All phenotypic analyses were done on plants grown under summer field conditions in Ames, IA. Plant height was measured from the bottom of the plant (the soil surface) to the top of the tassel. For internode length measurements, fully mature plants were dug from the ground and dissected. Nodes and internodes were numbered and counted starting from the first seedling leaf node at the bottom of the plant, progressing to the upper parts. Impressions of epidermal cells were obtained from internodes by painting the culm surface with clear nail polish. After drying, the nail polish was peeled and examined under an Olympus BX60 microscope using differential interference contrast (DIC). Impressions were digitally photographed with a Jenoptik C5 camera and cell lengths were measured with PROGRES 2.0 image analysis software.

Mature leaf blades and auricle tissues were prepared for histological examination by cutting into small pieces and fixing in FAA (5% formalin, 50% ethyl alcohol, 10% glacial acetic
acid). The dehydration and paraffin embedding protocol was as described in (Berlyn and Miksche, 1976). Paraffin blocks were sectioned at 7-10 μm with a Leica RM2235 manual microtome, sections affixed on glass slides, dewaxed, stained with 0.1 % Toluidine Blue and mounted with Permoun™.

**Kinematic analysis of leaf growth**

The kinematic analysis was performed as described in Nelissen et al., 2013. Leaf four was measured daily from appearance until growth stopped. By doing so, the leaf elongation rate (LER) was calculated (in mm/h). The first days of linear increase are considered as steady-state growth (Ben-Haj-Salah and Tardieu, 1995). Leaf four was sampled during steady state growth (two days after appearance) for DAPI staining and differential interference microscopy to determine the cell length profiles. The growth analyses (LER and final leaf length) were done on at least five plants per genotype and the kinematic analysis is performed on three plants per genotype.

**BES1-YFP response to Brassinolide (BL) in maize tissues**

Behavior of the BES1-YFP reporter in response to BL was examined in several tissues from maize lines expressing the transgene. These included leaf sheath, mature and developing auricle, and leaf blade. Tissues were exogenously treated with varying concentrations of pure BL prepared in 1X PBS buffer. A droplet of BL solution was applied to maize tissue (tissue was not submerged in solution) and BES1-YFP expression checked at various intervals over a time course with a fluorescence microscope. While all these tissues could be observed to respond, the most favorable for photomicrography was sheath tissue. Nuclear fluorescence within the pre-auricle band of developing leaves was measured using ImageJ as described (McCloy et al., 2014).
**BR root inhibition assay**

Surface sterilized seeds (70% EtOH followed by 5% bleach) were soaked in sterilized wet paper towel for 2-3 days until germination. Germinated seeds were transferred to treatment containers containing paper towels soaked with the BL treatment solution, 0, 20 or 100nM pure BL in ddH2O. Paper towels at the bottom of containers were re-wet with the appropriate BL solution every other day. Seedlings were grown under continuous light at room temperature for 7-8 days at which time each plant was genotyped for the presence of the Bar gene (marker for the transgene) and the primary root length was measured. Statistical analyses were done with the StatPlus application (AnalystSoft).

**Image analysis**

Images used for fluorescence quantification were captured by confocal microscopy at the ISU Confocal and Multiphoton Facility. Image analysis was done using (Fiji Is Just) ImageJ V:2.0.0-rc-23 software (Schindelin et al., 2012). For each nucleus, total YFP signal was calculated via corrected total cell fluorescence (CTCF) method (McCloy et al., 2014).

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caused by brassinosteroid deficiency increase biomass production and grain yield in rice. 

Nat Biotechnol 24: 105-109


**Figure Legends**

**Figure 1.** Characterization of BRI1 homologs in maize. A, BRI1 protein domains. The region of BRI1a used for RNAi is indicated. B, Phylogenetic analysis showed there are 5 BRI1 homologs in maize, including 3 brl genes and a maize-specific duplication of the bril gene. C, Island domain (ID) and LRR22 domain alignment of BRI1 homologs show that all maize homologs belong to the BRI1 family. D, qRT-PCR expression analysis of BRI1 homologs in zmbr11-RNAi plants. bri1-RNAi(s) is the strong event, bri1-RNAi(m) a mild event. Bars show proportional expression of the genes in RNAi lines relative to WT siblings with WT expression set to 1.0.
**Figure 2.** *bri1*-RNAi plants have disrupted BR signaling. A, BR marker gene expression in strong *bri1*-RNAi plants. Bars show proportional expression of genes in RNAi lines over WT siblings with gene expression set to 1.0 in WT. B, BL root growth inhibition assay. Lines represent relative root length of BL treated roots over mock treated. D, BES1-YFP expression pattern in WT and *bri1*-RNAi leaf sheath tissue. In WT, BES1-YFP accumulation in nuclei of leaf sheath cells could be induced to higher levels by BL application. The *bri1*-RNAi cells showed decreased BES1-YFP nuclear expression in untreated tissue, and showed a slower response to exogenous BL treatment.

**Figure 3.** *bri1*-RNAi plant architecture. A, *bri1*-RNAi plants with a WT sibling. A close-up image of a *bri1*-RNAi plant is on right top corner. B and C, Dissected *bri1*-RNAi plants show extremely shortened internodes clustered between the ear and tassel nodes. D, Internode length measurements of *bri1*-RNAi plants. Bars show proportional internode length of the strong *bri1*-RNAi plants over WT siblings. While early internodes are moderately shortened, later internodes are strongly affected (X axis represents internodes; Y axis represents proportional internode length of transgenic lines). E, Internode epidermal cells are shortened in both mild and strong *bri1*-RNAi lines. Bars show proportional internode epidermal cell length of *bri1*-RNAi plants over WT siblings. Epidermal cells are shortened 40% in the mild line, and ~70% in the strong *bri1*-RNAi line compared to WT siblings. F, WT internode epidermal cells (size bar 100 μm). G, *bri1*-RNAi internode epidermal cells (size bar 100 μm).

**Figure 4.** *bri1*-RNAi inhibits leaf growth. A, WT versus *bri1*-RNAi leaves of greenhouse grown plants. B, leaf epidermal cells of WT. C, *bri1*-RNAi show decreased cell elongation. D, and E, Mature leaf blade sections show that *bri1*-RNAi leaves (E) are thicker than WT leaves. *bri1*-RNAi leaf epidermal cells are enlarged in cross section, even though they are shorter in length.
Kinematic analysis showed that leaf elongation rate is decreased in *bri1-RNAi* during steady-state growth (days 1-5 after leaf emergence).

**Figure 5.** *bri1-RNAi* disrupted leaf auricle formation. A, Auricle (bracket) of a WT leaf. B, *bri1-RNAi* leaves lacking well defined auricles. C, Adaxial view of leaves. WT is on the left showing a normal auricle (bracket) and ligule (arrow). The *bri1-RNAi* leaf (right) lacks a normal auricle and has a reduced ligule (arrow). D and E, Confocal images showing nuclear BES1-YFP protein accumulation in a band of cells at the ligule-auricle region in developing plastochron 7 leaves. Signal intensity in the “auricle band” of WT (D) was 47% higher than band of *bri1-RNAi* (E). The auricle band of *bri1-RNAi* (E) was narrower and nuclear fluorescence intensity was less bright compared to WT band (D).

**Figure 6.** *bri1-RNAi* plants (left) resemble *Rs1-O* mutants (right).

**Supplemental Figure S1.** Amino acid sequence alignment of BRI1 homologs from Arabidopsis (At), rice (Os) and maize (Zm).

**Supplemental Figure S2.** Transcript levels of maize *bri1* homologs in B73 maize tissues. Data were retrieved from the Maize Gene Expression Atlas (Sekhon et al., 2011) using the eFP tool (Winter et al., 2007) available through MaizeGDB (Monaco et al., 2013). The histogram contains data from the following subset of available samples that were chosen to survey expression patterns: (anthers, immature cob, immature tassel stage v13, primary root stage v1, shoot apex stage v4, internode 4 stage v9, tip expanding leaf stage v7, base expanding leaf v7, silks stage r1, whole kernel 2 DAP, embryo 16 DAP, endosperm 16 DAP, pericarp 16 DAP, and germinating seed 24 hr after radicle emergence). The dotted line represents the gene expression cutoff value below which the authors consider a gene as not expressed.
**Supplemental Figure S3.** A, Transcript levels of maize BRI1 homologs in developing B73 maize seedling leaf 3. Data were retrieved from publically accessible RNAseq transcriptomic analysis along the maturation gradient of a growing leaf (Wang et al., 2014). RNA was extracted from 1cm segments beginning at the base of the leaf (M1) and going to the tip (M15). The base is the meristematic zone of active cell division whereas tissues at the tip are fully differentiated and mature. B, Relative transcript levels in 1 cm increments along maize seedling leaf 4 as determined by quantitative reverse transcriptase polymerase chain reaction.

**Supplemental Figure S4.** Nucleotide sequence of the zmbri1a cDNA used for RNAi construct aligned with BRI1 homologs. Nucleotide base 1 of the RNAi fragment corresponds to base 721 of Zmbri1a, 745 of Zmbri1b, 1180 of zmbrl1, 751 of zmbrl2, and 919 of zmbrl3.

**Supplemental Figure S5.** BR root growth assay. RNAi lines have decreased BR sensitivity compared to WT siblings. 100nM BL treated WT seedlings’ primary root length was ~40% decreased compared to untreated WTs, while primary root length of BL treated RNAi plants is 20% decreased compared to untreated RNAi seedlings.

**Supplemental Figure S6.** Internode length of mild bri1-RNAi plants. Bars show proportional internode length of the mild bri1-RNAi plants as a percentage of the corresponding internode in non-transgenic siblings. There is a fairly uniform decrease over all internodes in RNAi lines, except a more pronounced shortening of internodes 9 and 10 just past the ear node.

**Supplemental Figure S7.** Leaf traits of bri1-RNAi plants. A, bri1-RNAi dwarves show characteristic wavy margins producing the appearance of a corkscrew twist. B, Compared to WT (left), strong bri1-RNAi leaves have a wrinkled surface and are dark green in color. C and D, Histological sections through the auricle regions of WT (C) and bri1-RNAi (D) leaves. RNAi
leaves do not show normal auricle anatomy, with thickened tissue, enlarged vascular bundles and enlarged cortical parenchyma (size bar 100 μm).

Tables

Table 1. Sequence homology comparisons among maize BRI1-related proteins: % identity (% similarity)

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<tr>
<th>Gene Model/ Genbank accession</th>
<th>AtBRII</th>
<th>OsBRII</th>
<th>ZmBRIIa</th>
<th>ZmBRIIb</th>
<th>ZmBRL1</th>
<th>ZmBRL2</th>
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<td>79 (88)</td>
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<td>ZmBRIIb</td>
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<td>79 (88)</td>
<td>93 (95)</td>
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<td>—</td>
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<td>ZmBRL1</td>
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<td>47 (60)</td>
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<tr>
<td>GRMZM2G002515/XM_008660956</td>
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<td>43 (58)</td>
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<td>GRMZM2G438007/XR_565429</td>
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<td>46 (62)</td>
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<td>44 (59)</td>
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Table 2. Phenotypic analyses of RNAi lines*

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<tr>
<th></th>
<th>Node Count</th>
<th>Blade length (cm)</th>
<th>Blade Width (cm)</th>
<th>Leaf sheath length (cm)</th>
<th>Plant Height (cm)</th>
<th>Flowering time (days)</th>
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<td>Wt</td>
<td>16.5±0.3</td>
<td>75.25±0.9</td>
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<td>14.15±0.2</td>
<td>230.7±7.4</td>
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<td>p-value</td>
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<td>0.00067</td>
<td>0.45</td>
<td>2.95286E-05</td>
<td>0.008</td>
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<td>RNAi-mild lines</td>
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<td>15±0</td>
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<td>Wt</td>
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* p-values are based on Student’s t-test statistical analysis.
# : Since most of the mutants have enclosed tassel, no accurate data has gotten for this analysis.
### Table 3. Kinematic growth analysis of leaf #4

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<th>bri1-RNAi</th>
<th>% difference</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Size division zone (cm)</td>
<td>1.98 ± 0.08</td>
<td>1.43 ± 0.08</td>
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<td>Size dividing cells (μm)</td>
<td>26.77 ± 0.31</td>
<td>23.39 ± 0.87</td>
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<td>Number of dividing cells</td>
<td>799.54 ± 32.45</td>
<td>634.47 ± 50.37</td>
<td>-26.02</td>
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<tr>
<td>Leaf elongation rate (mm.h⁻¹)</td>
<td>2.81 ± 0.09</td>
<td>2.41 ± 0.07</td>
<td>-16.79</td>
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<td>Cell division rate (cell. Cell⁻¹ h⁻¹)</td>
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<td>0.314</td>
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<td>Cell cycle duration (h)</td>
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<td>24.54 ± 2.70</td>
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<td>Mature cell length (μm)</td>
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<td>134.14 ± 7.71</td>
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<tr>
<td>Final leaf length (mm)</td>
<td>606.5 ± 11.02</td>
<td>554 ± 17.58</td>
<td>-9.48</td>
<td>0.068</td>
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### Table S1. Fluorescence intensity of nuclei in the auricle bands of WT vs. zmbri1-RNAi leaves.

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<td>1</td>
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<td>6949.1 (246.6)</td>
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<td>2</td>
<td>10131.7 (327.5)</td>
<td>8332.2 (302.6)</td>
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<td>3</td>
<td>1529.7 (90.0)</td>
<td>805.9 (46.2)</td>
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Table S2. List of primers and sequences used

<table>
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<th>Construct</th>
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<td>BES1-YFP-promoter&amp;genomic coding sequence-</td>
<td>BES1_3G Wp1</td>
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<td>GGGGACAAGTTTGTACAAAAAGCAGGCTGCATTGA TGCTATCGGAGAT</td>
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| Primers used for genotyping transgenic lines   | Bri1-RNAi | Waxy-gk1 | CCAGTTCAAAATTCTTTTAGGCTCACC |
|                                               |          | Mu278-F  | GAACAAACTACCTCTCCGGCG       |
| BES1-YFP                                      | Bes1yfp-F2 | TGGATCAGCTCCAGGCCACCA |
|                                               | Bes1yfp-R | GAAGAAGTCGTGCTGCCTACATGTG |

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<th>Primers used for Quantitative PCR</th>
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<table>
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<td>DAA46648</td>
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<tr>
<td>ZmBRL3</td>
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Figure 1. Characterization of BRI1 homologs in maize.
Figure 2. *bri1*-RNAi plants have disrupted BR signaling
Figure 3. *bri1*-RNAi plant architecture.
Figure 4. *bri1*-RNAi inhibits leaf growth.
Figure 5. *bri1*-RNAi disrupted leaf auricle formation.
Figure 6. *bri1*-RNAi plants (left) resemble *Rs1-O* mutants (right).
Supplemental Figure S1. Amino acid sequence alignment of BRI1 homologs from Arabidopsis (At), rice (Os) and maize (Zm).
**Supplemental Figure S2.** Transcript levels of maize bri1 homologs in B73 maize tissues
Supplemental Figure S3. A, Transcript levels of maize BRI1 homologs in developing B73 maize seedling leaf 3.
Supplemental Figure S4. Nucleotide sequence of the zmbr1la cDNA used for RNAi construct aligned with BRI1 homologs.
Supplementary Figure S5. BR root growth assay

Supplementary Figure S6. bri1-RNAi (mild) internode length measurements
Supplementary Figure S7. Leaf traits of bri1-RNAi plants.
CHAPTER 3. SUPPRESSION OF GSK3/BIN2 FAMILY BY RNAi APPROACH CAUSES ALTERED BRASSINOSTEROID SIGNALING AND HAS PLEIOTROPIC EFFECTS IN MAIZE (ZEA MAYS) GROWTH AND DEVELOPMENT

A paper to be submitted to Plant Journal

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Abstract

Brassinosteroid hormones are involved in many developmental processes such as cell elongation, cell division, leaf and root development. Despite being well-established in model organisms, the BR signaling pathway is not well understood in maize (Zea mays), which is an important crop worldwide. In order to investigate BR signaling in maize, we used RNAi to target BIN2, a GSK3-like protein kinase that acts as a negative regulator of BR signaling. BLAST searches and further analyses identified a total of ten GSK3 like kinases in maize, five of which are in the same clade as BIN2. Of the eight homologs examined, all had decreased expression in
RNAi lines. Suppression of BIN2 revealed novel phenotypes not reported in other species. Unexpectedly, RNAi lines were shorter than their wild type (WT) siblings due to shorter stem internodes. In contrast, male inflorescence internodes were elongated in RNAi lines making tassel branches longer. Leaf blades were elongated and wider with crenulated margins, leaf sheathes were elongated, and auricles expanded. In addition, bin2-RNAi plants show some interesting kernel traits including increased embryo size and opaque endosperm with enlarged malformed starch grains. bin2-RNAi plant phenotypes appeared epistatic to those of bri1-RNAi transgenic lines, consistent with their relative functions in BR signaling.

**Introduction**

Brassinosteroids (BRs) are a class of plant hormones, which have various roles in plant development. They are involved in flower, root, and leaf development, cell elongation and division, biotic and abiotic stress responses and many other processes in a plant life cycle (Yamamuro et al., 2000; Catterou et al., 2001; Yin et al., 2002; Krishna, 2003; Mussig et al., 2003; Li et al., 2010; Ye et al., 2010). BRs were classified as plant hormones in mid-90s and since then intensive work on model organisms has identified key players of BR signaling (Li and Chory, 1997; Li et al., 2001; Wang et al., 2002; Yin et al., 2002; Vert and Chory, 2006; Wang and Chory, 2006; Gampala et al., 2007; Tang et al., 2008; Kim and Wang, 2010).

Despite their pervasive involvement in so many important aspects of plant biology, little work has addressed BR functions in maize, one of the world’s most important crops. There are two characterized maize mutants so far which have defects in BR biosynthesis. One of them is a classical maize mutant *nana plant1 (na1)* and the other one is *brd1*. *na1* has a mutation in a gene homologous to *DET2* encoding a 5α-reductase and *brd1* mutants have a nonsense mutation in *BRD1* gene encoding brassinosteroid C-6 oxidase (Hartwig et al., 2011; Makarevitch et al.,
Both mutants are dwarf with shortened internodes. A striking phenotype of these mutants was the feminized tassel, showing that BR biosynthesis is involved in sex determination in maize. In addition, leaf morphology was changed in these mutants. While there are two characterized BR biosynthesis mutants, there is no cloned maize BR signaling mutant yet. In a previous study to understand BR signaling’s role in maize development, we generated transgenic lines that had suppressed \textit{BRI1} expression. Similar to other BR mutants in maize and other species, \textit{bri1}-RNAi lines were also dwarf and had shortened leaves with disrupted blade/sheath boundary and altered auricle morphology.

\textbf{BIN2} is a GSK3-like protein kinase that is a key control point central to regulating BR signaling activity. It functions as a negative regulator of BR signaling by inhibiting BR transcription factors including BES1, BZR1 and DLT1 (Wang et al., 2002; Yin et al., 2002; Tong et al., 2012). In the absence of BRs, BIN2 is active and phosphorylates these transcription factors to exclude them from the nucleus and target them for proteasomal degradation (He et al., 2002; Yin et al., 2002; Vert and Chory, 2006; Peng et al., 2008; Kim and Wang, 2010; Ryu et al., 2010; Tong et al., 2012). Without BRs, the BRI1 receptor kinase is inhibited by BKI1 at the cell membrane. Binding of BRs to BRI1 activate the receptor and cause BKI1 dissociation (Wang and Chory, 2006). BRI1 then interacts with the co-receptor BAK1 and they trans-phosphorylate each other, which increases BRI1’s activity (Li et al., 2002). Activated BRI1 phosphorylates BSK1 and CDG1 and these kinases phosphorylate and activate BSU1, a phosphatase containing a kelch-repeat domain (Tang et al., 2008; Kim et al., 2011). Activated BSU1 dephosphorylates and inactivates BIN2, and this, combined with the action of PP2A, leads to dephosphorylation of BES1/BZR1 transcription factors resulting in their nuclear accumulation (Mora-Garcia et al.,
These transcription factors control many downstream genes and regulate plant development (Yu et al., 2011). Glycogen synthase kinase 3(GSK3)/SHAGGY-like kinases (GSKs) are serine/threonine kinases which are represented by two members in animals and by multiple members in plants (Cohen and Frame, 2001; Yoo et al., 2006; Rayasam et al., 2009; Qi et al., 2013). In animal systems, GSK3 kinases have been shown to negatively regulate many pathways by phosphorylation (Cohen and Frame, 2001). GSK3s have various roles in plant development including cell expansion, hormone signaling, stomata development, floral development, and embryo development (Piao et al., 1999; Dornelas et al., 2000; Li and Nam, 2002; Tavares et al., 2002; Claissse et al., 2007; Kim et al., 2012), as well as functions in biotic and abiotic stress responses (Piao et al., 1999; Christov et al., 2014).

BIN2 was first identified as a semidwarf Arabidopsis mutant, which was insensitive to exogenous BL, the most active BRs in Arabidopsis (Li et al., 2001). Gain-of-function bin2 mutants and transgenic plants with overexpressed BIN2 show BR insensitivity whereas plants with reduced expression of BIN2 show increased BR signaling (Li et al., 2001; Li and Nam, 2002; Rozhon et al., 2010; Tong et al., 2012). Arabidopsis gain-of-function bin2 mutants with compromised BR signaling have phenotypes, which includes darker green color, stunted growth, and late flowering (Li et al., 2001; Li and Nam, 2002). Arabidopsis plants with suppressed BIN2 homologs have elongated and wavy petioles and narrow leaves (Yan et al., 2009). In rice, BIN2 overexpression plants showed BR insensitivity with phenotypes including dark green and erect leaves, dwarf plants with less tillers, and shorter blade and sheath length (Tong et al., 2012). Suppression of BIN2 in rice caused plants with increased BR signaling, increased lamina joint bending, elongated narrowed leaves, and longer seeds (Tong et al., 2012).
There are ten GSK3-like kinases in Arabidopsis and nine in rice (Yoo et al., 2006). While kinase domains of Arabidopsis and rice GSK3s are well conserved, their N and C-terminals show variability, which might be important for different functions of these GSK3s (Jonak and Hirt, 2002). Phylogenetic analyses of Arabidopsis GSK3s put them into four clades (Yoo et al., 2006). Among these, all clade II members function redundantly in BR signaling with BIN2 being the predominant contributor (Yan et al., 2009). In rice, four GSK3 are grouped with Arabidopsis clade II members (Yoo et al., 2006). In Arabidopsis, a triple mutant of the three clade II GSK3s, bin2;bil1;bil2, resembled bes1-D or bzr1-D gain of function mutants with constitutive BR signaling: elongated, wavy petioles and narrow twisted leaf blades (Yan et al., 2009). Yet, this triple mutant still responded to BRs, showing it was not saturated for BR signaling and implying that other GSK3 members might be involved (Yan et al., 2009). In support of this, studies showed that ASKθ, a clade III member, and all clade I members are involved in BR signaling (De Rybel et al., 2009; Kim et al., 2009; Rozhon et al., 2010; Saidi et al., 2012; Youn et al., 2013; Ji-Hyun and Kim, 2015).

To date, no study has focused on GSK3 functions in maize BR signaling. To address this, we followed a transgenic approach by knocking down GSK3 members with RNAi. Interestingly, RNAi plants were semi-dwarf, while we were expecting plants with promoted plant height because of increased BR signaling. Additionally, transgenic lines showed other novel phenotypes including elongated tassel internodes, long pedicels, longer and wider leaf blades with crenulated margins, enlarged auricles, altered kernel morphology and loose endosperm texture.
Results

BIN2 homologs in maize and the generation of an RNAi construct

Arabidopsis BIN2 protein sequence was used in database searches to identify maize GSK3 homologs. Similar to Arabidopsis and rice, maize contains 10 genes that encode GSK3-like proteins (Fig. 1). Phylogenetic analyses showed that there are five maize GSK3 (ZmBIN2) homologs contained in clade II (Figure 1). This clade contains three Arabidopsis homologs, including BIN2, which are the major GSK3s involved in BR signaling. Whereas Arabidopsis contains two GSK3 homologs in clade III, maize does not appear to contain any members of this group. In addition, clade I contains four maize members compared to three in rice and three in Arabidopsis, while clade IV contains one member in maize, one in rice and two in Arabidopsis.

Conserved regions of these GSK3s are shown in Figure 2A. The Y200 residue, whose phosphorylation is required for fully active BIN2, is conserved in all maize GSK3s. Another important motif, TREE, is also conserved among all members except for GRMZM2G075992, where the first Glutamic acid (E) is changed to Aspartic acid (D). CDFGSAK which is only present in the GSK3 sub family of Serine/Threonine protein kinases is also conserved among all members. A SIDIW domain is only present in clade II members and is conserved in five of the maize GSK3 homologs.

We checked the expression of GSK3 homologs in different maize tissues by utilizing publicly available databases (Sekhon et al., 2011). Most of the homologs are being expressed universally throughout the plant during growth and development, while some of them are being expressed in some specific tissue types. For example, GRMZM2G121790 and GRMZM2G045330 are being expressed highly in all tissue types. In contrast, GRMZM2G075992 is mainly expressed in silk. Expression profiles of GSK3 homologs are shown in Figure S3 and Table S1.
To overcome the likely functional redundancy among maize BIN2 homologs, an RNAi strategy was undertaken to knock down as many as possible. The full-length cDNA sequence of the top candidate BIN2 homolog was used for an RNAi construct under the control of the maize ubiquitin promoter. The construct was introduced into HiII maize by Agrobacterium-mediated transformation and backcrossed to B73 and W22 inbred lines. Expression of the GSK3 genes in \textit{bin2}-RNAi lines was examined with RNA isolated from developing leaves. Of the eight homologs that are expressed in the leaf, all showed decreased expression based on quantitative-PCR (qRT-PCR) (Figure 2B).

\textbf{\textit{bin2}-RNAi effects on plant architecture}

As a negative regulator of BR signaling, reduced BIN2 expression was expected to cause increased plant growth. However, in seven independent RNAi events, plants were shorter compared to their non-transgenic counterparts (Figure 3A). The semi-dwarf phenotype appeared to be primarily due to decreased stalk internode length, whereas internode number did not significantly vary between RNAi and WT segregants (Table 1). Internodes of RNAi lines were shortened in a consistent manner along the whole stalk (Figure 3B and 3C). As seen Figure 3D-F, epidermal cells of internodes are shorter in RNAi lines indicating that decreased cell elongation contributed to the reduced internode length.

Although stem internodes of \textit{bin2}-RNAi plants were shortened, tassel inflorescence internodes were elongated, producing an elongated central spike and tassel branches, and contributing to lower spikelet density (Fig. 4, Table 1). Furthermore, spikelet pair pedicels were elongated (Figure 4D). In addition, the tassel main spike and branches are often undulate (Figure 4B). The main spike and branches were also often barren near the tips suggesting abortion or failure to initiate spikelet pairs in \textit{bin2}-RNAi plants (Figure 4A).
Leaf morphology was dramatically affected on bin2-RNAi plants. Blade length and width of transgenic lines was significantly greater than WT siblings (Figure 5A). Blades also had a crinkly texture with striking crenulated margins (Figure 5A and 5B). In very strong events, leaves failed to unfurl normally resulting in severe growth defects and failure of tassel emergence (Figure S5). Husk leaves were also longer in RNAi lines. Perhaps the most significant aspect of the leaf phenotype in RNAi lines was the expanded auricle, giving bin2-RNAi leaves a less upright habit (Figure 5E). These morphological effects become much more pronounced in adult leaves than on seedling leaves.

In addition to these morphological phenotypic characteristics, bin2-RNAi plants flower late compared to their WT siblings (Table 1).

**bin2-RNAi plants have altered BR signaling**

A BR root inhibition assay is a commonly used method to check plants’ BR signaling status. While exogenously applied BRs show a dose-dependent inhibition of root growth in wild type plants, there is decreased inhibition in plants with compromised BR signaling (Clouse, 1996; Chono et al., 2003; Mussig et al., 2003). Roots were treated with 100 nm pure BL for six times every other day after germination. As seen in Figure S1, WT plant root growth was inhibited by BR treatment. Interestingly, untreated bin2-RNAi roots were shorter than untreated WT siblings, whereas there was no significant change in root growth of the bin2-RNAi line upon BR treatment. These results suggest an altered BR signaling status in bin2-RNAi seedlings.

Several genes encoding enzymes in BR biosynthesis are negatively feedback regulated by BR signaling. Decreases in BR signaling cause increased expression of these genes, while higher BR signaling leads to decreased expression (Mathur et al., 1998; Hong et al., 2002; Bai et al.,
Expression of such BR marker genes was assayed in bin2-RNAi lines, but significant changes in expression were not observed (Figure S4).

Previously, bri1-RNAi lines were shown to have impaired levels of BR signaling (Kir et al.). According to the established BR signaling pathway, BIN2 functions as a negative regulator of BR signaling downstream of BRI1. As such, disruption of BIN2 function would be expected to suppress phenotypes associated with BRI1 inhibition, as was shown in rice (Yan et al., 2009). To test this hypothesis, bin2-RNAi and bri1-RNAi lines were crossed together and their phenotypes examined. We found that bin2-RNAi lines partially or sometimes completely rescued the bri1-RNAi phenotypes. Knock down of BRI1 resulted in pleiotropic effects on maize development, including shortened internodes, small crinkly leaves and decreased auricle size. The bin2-RNAi; bri1-RNAi double transgenic lines generally looked more like bin2-RNAi lines than bri1-RNAi.

The clearest result of the double transgenic lines was the rescued auricle phenotype of bri1-RNAi lines. bri1-RNAi lines have disrupted auricle morphology, with auricles mostly absent in later stage leaves (Figure 7B). In contrast, bin2-RNAi lines have larger auricles (Figure 7B). bin2-RNAi completely or partially suppressed the auricle phenotype of bri1-RNAi lines. Leaf length phenotypes of double mutant lines also more closely resembled bin2-RNAi as did blade texture and color, tassel and ear morphology (Figure 7 and Table-3). In conclusion, most of the bri1-RNAi phenotypes were rescued either partially or near fully by bin2-RNAi. Not only does this suggest that BIN2 is located downstream of BRI1 in maize, but also that there is an increased level of BR signaling in bin2-RNAi lines. Plant height phenotypes were somewhat ambiguous. While strong bri1-RNAi plant height was partially rescued by bin2-RNAi transgenic lines, plant height of mild bri1-RNAi lines was not different from double lines (Table-3).
total, the suppression of many bri1-RNAi phenotypes by bin2-RNAi is consistent with the functions of BIN2 and BRI1 in BR signaling and suggests that bin2-RNAi plants have increased levels of BR signaling.

**Kinematic analysis of bin2-RNAi leaves**

To understand the developmental basis of the longer leaf phenotype, a kinematic growth analysis was performed on developing 4\textsuperscript{th} seedling leaves (Figure 5D and Table-2). Both leaf elongation rate (LER) and final leaf length were increased 14% in RNAi lines. The size of the division zone and number of dividing cells were both increased in bin2-RNAi lines. Mature cell length was not significantly different between WT and bin2-RNAi epidermal cells of leaf 4, indicating that the primary contributor to increased organ length was an increased number of cells. Interestingly, in adult leaves with more pronounced phenotypic effects, mature cell length is actually shorter in bin2-RNAi than WT, suggesting that the increased cell number becomes more accentuated later in development.

**BIN2 and maternal regulation of kernel traits**

Kernels borne on bin2-RNAi plants showed several striking phenotypes. The crown regions tapered to spiny points at the silk scar (Figure 6), which is distinct from the dent morphology typical of the B73 recurrent parent. bin2-RNAi kernels are also larger and have bigger embryos. In addition, the endosperm was opaque, floury in texture, with very little vitreous endosperm (Figure 6B and 6C). These traits are conditioned by the maternal sporophyte. Exact reciprocal crosses were performed between bin2-RNAi / - hemizygotes and B73 WT. When bin2-RNAi was used as a male, all the kernels showed normal phenotypes typical of B73. When used as a female, all the kernels showed the unusual shape and opaque texture, even though only half the kernels inherited the bin2-RNAi transgene.
Normal vitreous endosperm is conditioned by a semi-crystalline matrix formed through interactions between starch grains and zein storage protein bodies. Alterations in either zein protein content or starch structure can confer opaque endosperm. Zein storage protein content was profiled by HPLC, however no obvious differences were observed between kernels borne on the B73 WT versus bin2-RNAi females (not shown). Scanning electron microscopy (SEM) revealed that opaque kernels contained loosely packed starch granules that were larger and irregular in shape, compared to the spherical starch grains tightly embedded in a cellular matrix (Figure 6F and 6G). Hence, BIN2 signaling in the maternal tissues somehow conditions starch deposition in endosperm.

Discussion

GSK3s are involved in many developmental processes in both animals and plants (Cohen and Frame, 2001; Claisse et al., 2007; Yan et al., 2009). In plants, they function in hormone signaling, floral development, cell expansion, as well as biotic and abiotic stress responses (Piao et al., 1999; He et al., 2002; Li and Nam, 2002; Perez-Perez et al., 2002; Claisse et al., 2007; Yan et al., 2009; Rozhon et al., 2010; Kim et al., 2012; Christov et al., 2014). Despite their importance in diverse processes of agricultural significance, they have not been studied in maize. To address this, we undertook a transgenic RNAi suppression analysis to explore GSK3 functions, focusing on their role in development and BR signaling.

GSK3 family in maize

GSK3s are represented by 10 members in Arabidopsis and 9 in rice (Yoo et al., 2006; Qi et al., 2013). BLAST searches identified 10 possible GSK3 homologs in maize. GSK3 family in Arabidopsis was grouped into four clades, with all clade II members being involved in BR signaling (Yoo et al., 2006; Qi et al., 2013). Recent evidence indicates clade I involvement in BR
signaling as well (De Rybel et al., 2009). Maize GSK3s clustered with Arabidopsis homologs under all clades except clade III that lacked maize homologs. In Arabidopsis there are three GSK3s under clade II, four in rice, and 5 in maize. All of these 5 homologs had SIWID Domain, which is only present in clade II GSKs (Figure 2A) (Bittner et al., 2013). This suggests maize could have a higher level of redundancy among clade II GSK3s that perform the primary functions in BR signaling. Clades I and IV also show variations in membership and gene relationships among species suggesting the potential for functional diversification.

*bin2-RNAi have pleiotropic effects on maize development*

Although the RNAi construct was generated based on a clade II gene, all the leaf-expressed homologs were also affected (Fig. 2B). Reduced expression of this gene family had pleiotropic effects on maize development. There was a striking reduction in plant height among RNAi lines, which was unexpected for a negative regulator of BR signaling. Since BRs promote plant growth, the prediction was for increased plant height. There was no difference in node numbers but internodes contained smaller epidermal cells compared to their non-transgenic counterparts (Figure 3) suggesting that decreased cell elongation contributed to the decreased height. Given the results of the kinematic analysis of leaf growth (Figure 5D), it seems likely that changes in cell proliferation also contributed to the altered growth of internodes, although that remains speculative at this point. Interestingly, while stem internodes were shorter, tassel branches of RNAi lines were longer than WT siblings, due to elongated internodes. Also, spikelet pedicels and spikelet pairs of RNAi lines were longer (Figure 4). Thus, different internodes responded differently to the RNAi lines. These effects could be due to differential expression of the GSK3 family members, differential expression of the RNAi transgene, or differential BR levels, responses or sensitivities among tissues.
Another interesting phenomenon was the empty branch tips of RNAi tassels suggesting failure to initiate or early abortion of spikelet pair meristems (Figure 4A). This was reminiscent of auxin mutants (Barazesh et al., 2009). Also maize lazy plant1 (la1) mutants have tassels with empty branch tips. Dong et al. suggest that ZmLA1 might regulate auxin transport (Dong et al., 2013). Since it has been well documented that BRs cross-talk with auxin (Bao et al., 2004; Vert et al., 2008; Vanstraelen and Benkova, 2012), it is feasible that bin2-RNAi could be affecting some aspect of auxin levels or responses.

Leaves of RNAi lines were longer and wider than their WT siblings (Figure 5A). In rice, suppressed BIN2 expression changed the leaf morphology as well. Leaf elongation was also seen in rice plants with reduced expression of OsGSK2 but in contrast to maize, blade width of rice lines was narrower (Tong et al., 2012). This suggests that BR signaling has differential effects on directional growth in these two grasses. A strong effect on directional growth was observed in maize with suppressed BRII expression. Epidermal cells of bri1-RNAi leaves showed decreased length but were increased in the depth dimension (Kir et al. submitted). A kinematic analysis on leaf growth showed that leaf elongation rate (LER) was increased in bin2-RNAi lines and that leaves showed increased cell division and an extended division zone (Figure 5D) (Table 2). This is as expected, compared to bri1-RNAi lines which showed decreased rates of steady-state leaf elongation due to decreased cell proliferation and elongation (Kir et al., submitted). Leaves also showed crinkly texture with unusual crenulated margins, suggesting that growth coordination was compromised by bin2-RNAi (Figure 5A and B).

Auricles were enlarged on bin2-RNAi leaves, contributing to a less upright habit (Figure 5E). This is opposite what was observed on bri1-RNAi plants, which showed decreased auricle formation and more upright leaves. Leaf angle is an important aspect of the maize plant ideotype
with upright leaf habit critical for tolerance to the higher planting densities required for high yield in maize (Mock and Pearce, 1975; Duvick et al., 2004; Duvick, 2005; Ford et al., 2008). Lamina joint bending is also a well-known BR response in rice (Yamamuro et al., 2000; Tsuda et al., 2014).

**bin2-RNAi lines have increased levels of BR signaling**

There are five maize GSK3 homologs clustered with Arabidopsis Clade II GSK3s, which perform primary functions as negative regulators of BR signaling. qRT-PCR results show that all of these five homologs have reduced expression in RNAi lines (Figure 2B). As such, increased levels of BR signaling were expected. Some of the standard BR assays produced somewhat ambiguous results. Expression of BR marker genes was not significantly altered in leaf tissues. Root growth was decreased in the RNAi lines compared to WT but showed no change in response to exogenous brassinolide.

Perhaps the clearest evidence of functions in BR signaling is the opposite phenotypes between *bin2*-RNAi and *bri1*-RNAi lines. *bri1*-RNAi lines had shorter blades, decreased auricles and upright leaf habit. The *bri1*-RNAi lines showed clear evidence of impaired BR signaling including increased expression of BR feedback-inhibited genes, decreased sensitivity in root growth assays, and decreased levels of BES1-YFP reporter expression (Kir et al, submitted). Epistatic relationships between *bin2*-RNAi and *bri1*-RNAi lines were consistent with BIN2 functioning downstream of BRI1 in BR signaling. Double “mutant” leaves showed the enhanced growth, crinkled texture, crenulated margins and enlarged auricles conditioned by *bin2*-RNAi (Figure 7).

GSK3s, including BIN2 itself, are also known to have functions in processes other than BR signaling. These include functions in redox signaling, carbohydrate metabolism, biotic stress
responses, as well as many aspects of cell differentiation and development (reviewed in (Piao et al., 1999; Claisse et al., 2007; Christov et al., 2014; Ji-Hyun and Kim, 2015). Among Arabidopsis GSK3 members, AtSK11 and AtsK12 have higher transcript levels in floral organs and their inhibition causes disrupted flower growth with more numbers of petals and sepals (Dornelas et al., 2000). In addition, AtSK32, a group III member, also has localized expression in floral meristems and is involved in embryo development (Tavares et al., 2002). Several appear to function in multiple pathways; AtSK11 is also involved in stomata development in leaves, while AtSK21 (BIN2) also functions in auxin and ABA signaling, in stomata development, and in root hair development (Vert et al., 2008; Gudesblat et al., 2012; Kim et al., 2012; Khan et al., 2013; Cai et al., 2014; Cheng et al., 2014). MsK4 is a plastid-located GSK-3-like kinase involved in stress tolerance to high salinity in *Medicago sativa*. Plants that overexpressing MsK4 had higher amount of starch content under salt stress, which connects MsK4 to starch metabolism (Kempa et al., 2007). Taken together, it is highly possible that some aspects of the *bin2-RNAi* phenotype could be BR-independent. Sorting the specific functions among the different GSK3 family members is one of the challenges for future studies.

**Materials and Methods**

**Sequence analysis**

Using full length Arabidopsis BIN2 protein sequence with BLASTp (Altschul et al., 1990) on the NCBI refseq_protein database, 255 hits were returned with max scores ranging from 27.7 to 699, and identity from 35% to 88%. Among these, GI:212276029 was the highest with 699 max score, 98% query coverage, and 87% protein identity over the full-length BIN2 protein. From these, the top 12 hits were selected with max score ranging from 192 to 699 and amino acid identity from 35% to 88 %. All alignments were generated with ClustalW and
phylogenetic trees were obtained using Mega 6 NJ (Neighbor-joining) with p-distance model and partial deletion method (site coverage cutoff 95%) with applying 100 bootstrap.

Production of transgenic lines

The cDNA of gi: 212276029 (GRMZM2G121790_T01) was amplified by reverse transcription polymerase chain reaction (RT-PCR), cloned into pBluescript and sequenced at the Iowa State University DNA Facility. The full-length cDNA was introduced into the pMCG1005 vector used for the RNAi construct. Appropriate restriction sites were added to the primers ATGGCCGCGCATGCCGGGTGGGGCCCAC and TGGTCCAGTAGCGGGCAATAATGTGAG. These primers amplified bases from the start codon to 1236 bp. While StuI and AvrII enzymes were used to put the gene between Adhl intron and rice Waxy-a intron, SpeI and XmaI enzymes were used to put the gene in reverse orientation between OCS 3′ and rice Waxy-a intron. Expression of the construct is controlled by the maize ubiquitin1 promoter. The construct was introduced into HiII embryos by the Iowa State Plant Transformation Facility using Agrobacterium tumefactions based transformation. Transgenic calli were identified by Liberty herbicide resistance and regenerated to plants. Seven of seventeen regenerated lines showed consistent phenotypes as described in Results. Lines showing reproducible phenotypic effects were selected and backcrossed at least three times to B73 and W22 inbred lines.

Quantitative RT-PCR

Leaf tissues were frozen immediately in liquid nitrogen, ground and kept in -80 °C until RNA purification using a Qiagen RNeasy Mini Kit according to the manufacturer’s protocol. RNA concentrations were measured by a NanoDrop ND-1000 Spectrophotometer, samples DNase treated (Promega RQ1 RNase-free DNase) and 1-2 µg RNA used per experiment. 1µL
DNase and 1.2 µl RQ1 buffer were used and volumes adjusted to 12 µl with nuclease free water. Samples were incubated at 37°C for 30 minute, after which 1 µl RQ1 DNase stop solution was added and samples incubated at 65°C for 10 minutes. 11 µl of this volume was used directly for RT-PCR reactions using Invitrogen SuperScriptTM III Reverse Transcriptase according to the manufacturer’s protocol. From the 20µl cDNA reaction, 1-2 µl was used for qRT-PCR using iQ SYBR Green Supermix (BioRad) and gene specific primers (Table –S1) on Stratagene MX4000 instrument.

**Phenotypic analyses**

Each transgenic event was backcrossed a minimum of 3 times into one or more inbred genetic backgrounds, including B73 and W22. All phenotypic measurements were done on plants in B73 background, except for BR root inhibition assay for which plants in W22 background used. All phenotypic analyses were done on plants, which were grown under field conditions in Ames, IA. Plant height was measured from the bottom of the plant (the soil surface) to the top of the tassel. For internode length measurements, fully mature plants were dug from the ground and dissected. Nodes and internodes were numbered and counted starting from the first seedling leaf node at the bottom of the plant, progressing to the upper parts. Impressions of epidermal cells were obtained from internodes by painting the culm surface with clear nail polish. After drying, the nail polish was peeled and examined under an Olympus BX60 microscope using differential interference contrast (DIC). Impressions were digitally photographed with a Jenoptik C5 camera and cell lengths were measured with PROGRES 2.0 image analysis software.
**BR root inhibition assay**

Seeds were surface sterilized 3 min with 80% ethanol and 15 min in 3% sodium hypochlorite (two times with fresh sodium hypochlorite), rinsed with ddH2O and were soaked in sterilized wet paper towel for 2-3 days until germination. Germinated seeds were transferred to treatment containers containing paper towels soaked with the BL treatment solution, 0 or 100nM pure BL in ddH2O. Paper towels at the bottom of containers were re-wet with the appropriate BL solution every other day. Seedlings were grown under continuous light at room temperature for 12 days at which time each plant was PCR genotyped for the presence of the Bar gene or transgene and the primary root length was measured. Statistical analyses were done with StatPlus application (AnalystSoft).

**Kinematic analysis of leaf growth**

The kinematic analysis was performed as described in Nelissen et al., 2013. Leaf four was measured daily from appearance until growth stopped. By doing so, the leaf elongation rate (LER) was calculated (in mm/h). The first days of linear increase are considered as steady-state growth (Ben-Haj-Salah and Tardieu, 1995). Leaf four was sampled during steady state growth (two days after appearance) for DAPI staining and differential interference microscopy to determine the cell length profiles. The growth analyses (LER and final leaf length) were done on at least five plants per genotype and the kinematic analysis is performed on three plants per genotype.

**Acknowledgements**

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Figure Legends

Figure 1. GSK3 phylogeny in maize

Phylogenetic analysis showed 10 GSK3 homologs in maize: four clustered under Clade-I, five in Clade-II, and one in Clade-IV. None of the maize homologs cluster in Clade-III.

Figure 2. GSK3 homologs and their expression in RNAi lines

A. Conserved regions of GSK3 kinases in maize. Y200 is conserved among all homologs and the TREE domain is present in all except GRMZM2G075992. B. All GSK3 homologs examined had decreased expression in growing young leaf tissue collected from RNAi plants.

Figure 3. Plant architecture of bin2-RNAi lines.

A. RNAi plants (right) have reduced height compared to WT plants (left). B. Dissected WT (top) and bin2-RNAi culms (bottom). C. Relative internode lengths were similarly shortened throughout the bin2-RNAi culms compared to WT siblings. D-F. Culm internode epidermal cells of WT (D) and bin2-RNAi (E). F. Internode epidermal cells of transgenic plants are shorter compared to WT.

Figure 4. bin2-RNAi plants have altered tassel morphology.

A. bin2-RNAi tassels are barren of spikelets at the tips. B. The central spikes of RNAi lines (right) are longer and have undulate shape compared to WT (left). C. Branches of bin2-RNAi tassels (right) have increased angle compared to WT (left). D. Spikelet pedicels are longer in transgenic lines (middle and right) compared to WT (left). E. bin2-RNAi tassels have elongated internodes (bottom).

Figure 5. bin2-RNAi affects leaf morphology.

A-B. bin2-RNAi leaves are wider, longer, and have crenulated margins. C. WT and bin2-RNAi leaf epidermal cells. D. Steady state leaf elongation rate is increased in transgenic lines. E. bin2-RNAi plants have larger auricles
Figure 6. Maternal bin2-RNAi alters kernel morphology. Comparison of kernels from reciprocal crosses of bin2-RNAi and B73 WT. A. Kernel morphology, when bin2-RNAi used as female (top) and as male (bottom). B. Kernels of bin2-RNAi females (top) are often opaque compared to WT females (bottom). C. Normal vitreous kernels borne on WT females contain horny endosperm (arrows), which is decreased, or lacking in opaque kernels (last two) of bin2-RNAi females. D and E. bin2-RNAi female kernels show pointy shapes (right). F and G. Starch granules of opaque kernels (G) are larger, non-spherical and less compact compared to non-opaque kernels (F).

Figure 7. bin2-RNAi is epistatic to bri1-RNAi. A. Double mutants (right) more closely resemble single bin2-RNAi plants (left) than bri1-RNAi (middle). B. bin2-RNAi suppressed disrupted auricle of bri1-RNAi plants. C. bin2-RNAi suppresses bri1-RNAi short leaf phenotype. The double mutant (right) is similar to the bin2-RNAi single (center) and longer than bri1-RNAi (right). D. Double mutant tassel (right) resembles bin2-RNAi tassel (left) more than bri1-RNAi (center). E. bin2-RNAi (left: double, middle: single bin2-RNAi) suppresses bri1-RNAi (right) ear phenotype. F. bin2-RNAi crinkled leaf phenotype is epistatic to bri1-RNAi.

Figure S1. bin2-RNAi BR root assay. While, WT root growth is inhibited with exogenously applied BL, bin2-RNAi roots are less inhibited with BL treatment. Mock treated bin2-RNAi roots are shorter than WT plants as well.

Figure S2. Aminoacid alignment of ZmGSK3s. This alignment was used to generate phylogeny.

Figure S3. Expression profile of GSK3 homologs. Expression of GSK3 homologs in different maize tissues. Data retrieved from publicly available databases.
Figure S4. BR marker gene expression in RNAi lines. There was no significant change in terms of BRD1, CPD, and DWF4 expression between WT and RNAi lines.

Figure S5. A bin2-RNAi plant with strong phenotype.

Tables

Table 1. Phenotypic analyses of bin2-RNAi lines

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<th>Plant Height (cm)</th>
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<th>Blade length (cm)</th>
<th>Blade width (cm)</th>
<th>Main tassel length (cm)</th>
<th>Spikelet density</th>
<th>Flowering time (days)</th>
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<td>RNAi</td>
<td>121.7±4.4</td>
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Table 2: Kinematic growth analysis of leaf #4 of bin2-RNAi

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<td>Size division zone (cm)</td>
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<td>Size dividing cells (µm)</td>
<td>26.88 ± 2.5</td>
<td>27.37 ± 1.57</td>
<td>0.805</td>
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<tr>
<td>Number of dividing cells</td>
<td>833.84 ± 9.62</td>
<td>1026.27 ± 23.14</td>
<td>0.007</td>
<td>18.75</td>
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<tr>
<td>Leaf elongation rate (mm.h-1)</td>
<td>3.02 ± 0.10</td>
<td>3.33 ± 0.10</td>
<td>0.026</td>
<td>14.49</td>
</tr>
<tr>
<td>Cell production (cell.h-1)</td>
<td>20.27 ± 0.06</td>
<td>21.59 ± 0.47</td>
<td>0.104</td>
<td>6.12</td>
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<tr>
<td>Cell division rate (cell. Cell-1 h-1)</td>
<td>0.024 ± 0.0002</td>
<td>0.021 ± 0.0007</td>
<td>0.033</td>
<td>-15.43</td>
</tr>
<tr>
<td>Cell cycle duration (h)</td>
<td>28.52 ± 0.27</td>
<td>32.98 ± 1.12</td>
<td>0.05</td>
<td>13.55</td>
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<tr>
<td></td>
<td>Mature cell length (µm)</td>
<td>Final leaf length (mm)</td>
<td></td>
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<tr>
<td>----------------------</td>
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<td>------------------------</td>
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<td>WT</td>
<td>149.218 ± 0.46</td>
<td>657.43 ± 12.97</td>
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<tr>
<td>brial-RNAi</td>
<td>154.52 ± 3.30</td>
<td>762.33 ± 9.49</td>
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<tr>
<td>bril-RNAi:bin2-RNAi</td>
<td>0.248</td>
<td>0.0002</td>
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<td>bin2-RNAi</td>
<td>3.43</td>
<td>13.76</td>
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Table-3. Phenotypic analyses of brial-RNAi:bin2-RNAi plants.

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<th>bin2-RNAi</th>
<th>double</th>
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<tbody>
<tr>
<td>bin2-RNAi/brial-RNAi</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Plant height (cm)</td>
<td>175±9.9</td>
<td>54±8.2</td>
<td>116.5±5.6</td>
<td>69±11.6</td>
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<td>Blade length (cm)</td>
<td>85.6±1.3</td>
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<td>95.3±0.9</td>
<td>83.5±3.1</td>
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<td>Sheath length (cm)</td>
<td>15±1.3</td>
<td>4.3±0.1</td>
<td>17.7±1.4</td>
<td>9.5±0.2</td>
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<tr>
<td>bin2-RNAi/brial-RNAi</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Plant height (cm)</td>
<td>158.8±4.5</td>
<td>98.2±10.3</td>
<td>113.3±11.3</td>
<td>73.6±8.4</td>
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<tr>
<td>Blade length (cm)</td>
<td>74.8±2.3</td>
<td>68.7±1.1</td>
<td>90.1±4.2</td>
<td>78.8±4.8</td>
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<tr>
<td>Sheath length (cm)</td>
<td>15±0.4</td>
<td>7.2±0.5</td>
<td>17.1±1.6</td>
<td>13.3±0.3</td>
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Table S1. GSK3 expression profile in Sekhon atlas (Sekhon et al., 2011). *

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<tr>
<th>Gene</th>
<th>Primarily expressed in</th>
<th>Overall expression</th>
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<tr>
<td>GRMZM2G121790</td>
<td>Embryo, internode, leaves</td>
<td>High in all tissues</td>
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<tr>
<td>GRMZM2G151916</td>
<td>Immature tassel, shoot tip, root,</td>
<td>Mild expression in other tissues</td>
</tr>
<tr>
<td>GRMZM2G043350</td>
<td>Shoot tip, immature tassel, ear</td>
<td>Mild expression in other tissues</td>
</tr>
<tr>
<td>GRMZM2G045330</td>
<td>Leaves, internodes,embryo,ear</td>
<td>In most tissues expressed highly</td>
</tr>
<tr>
<td>GRMZM2G472625</td>
<td>Cob, silk, embryo, immature tassel,</td>
<td>Mild expression in other tissues</td>
</tr>
<tr>
<td>GRMZM2G138676</td>
<td>Internode, embryo, silk</td>
<td>Mild expression in other tissues</td>
</tr>
<tr>
<td>GRMZM2G075992</td>
<td>Leaves, tassel,</td>
<td>Mild expression in other tissues</td>
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<tr>
<td>GRMZM2G024151</td>
<td>Leaves, shoot tip</td>
<td>Mild expression in other tissues</td>
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* No eFP view exists for GRMZM2G155836 and GRMZM5G835235 in this expression database.
Table S2. List of primers and sequences used

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<th>Primer</th>
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Primers used for genotyping transgenic lines

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Primers used for Quantitative PCR

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Table S3. Accession number of genes used to create GSK3 phylogenetic tree

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<td>CAAGTAGGTAACCTTGACTCTTCA</td>
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</table>
Figure 1. GSK3 phylogeny in maize
Figure 2. GSK3 homologs and their expression in RNAi lines
Figure 3. Plant architecture of *bin2*-RNAi lines.
Figure 4. *bin2*-RNAi plants have altered tassel morphology.
Figure 5. *bin2-RNAi* affects leaf morphology.
Figure 6. Maternal bin2-RNAi alters kernel morphology.
Figure 7. *bin2-RNAi* is epistatic to *bri1-RNAi*
Figure S1. bin2-RNAi BR root assay.
Figure S2. Aminoacid alignment of ZmGSK3s.
Figure S3. Expression profile of GSK3 homologs.
Figure S4. BR marker gene expression in RNAi lines.
Figure S5. *A bin2-RNAi* plant with strong phenotype.
CHAPTER 4. GENERAL CONCLUSIONS

Brassinosteroids are plant hormones, which are involved in many developmental and physiological processes in the plant kingdom (Mandava, 1988; Clouse, 1996; Li et al., 1996; Li and Chory, 1997; Bishop, 2003; Gendron and Wang, 2007; Kim and Wang, 2010; Ye et al., 2011). Studies on model organisms such as Arabidopsis and rice revealed much about the BR signaling pathway (Clouse et al., 1996; Li and Chory, 1997; Yamamuro et al., 2000; He et al., 2002; Li and Nam, 2002; Wang et al., 2002; Yin et al., 2002; Wang and Chory, 2006; Bai et al., 2007; Gampala et al., 2007; Yan et al., 2009; Kim and Wang, 2010). However, BR signaling in maize is lacking much knowledge compared to these model organisms. There was no reported study on BR signaling in maize when we started this project.

Phylogenetic analyses showed that maize has two closely related BRI1 homologs and three BRI1-like genes, suggesting there was likely to be redundancy. To study the relatively unknown Brassinosteroid signaling (BR) in maize, we took a transgenic approach by targeting two members of BR signaling by RNAi: BRI1 and BIN2. BRI1 encodes for a receptor kinase, which is located on the cell membrane (Li and Chory, 1997), and BIN2 encodes for GSK3- Shaggy-like kinase, which acts as a negative regulator in the BR signaling pathway (He et al., 2002; Li and Nam, 2002). We analyzed the BRI1’s and BIN2’s role in maize development in Chapter 2 and Chapter 3, respectively.

Downregulation of BRI1 resulted in pleiotropic effects on maize growth and development. The first striking phenotype of bri1-RNAi plants was their dwarf stature. A shorter plant height is a desired agricultural trait, as it is shown in the Green Revolution that higher grain yield could be obtained with semidwarf crops (Peng et al., 1999; Khush, 2001). The shorter plant height phenotype of bri1-RNAi lines was due to shortened internodes. Interestingly, while there
was a steady decrease in early internodes’ length, later internodes were extremely shorter which possibly makes upper bri1-RNAi leaves less unfurled. Another effect of downregulation of BRI1 was on leaf development of maize. Leaves of bri1-RNAi plants were shorter, dark green in color, thicker, and erect. Another agriculturally desired trait of bri1-RNAi is their upright leaf phenotype. Similar to shorter plant height, upright leaf phenotype is also a desired trait, because it increases light penetration in higher density canopy. The auricle is a hinge like element, which determines the angle of maize leaves. Decreased or sometimes absent auricles caused an upright leaf phenotype in bri1-RNAi lines. A BR marker, BES1: YFP, showed a high accumulation in the developing ligule/auricle region, which also suggests that BR signaling is involved in auricle development.

In Chapter 3, generation of bin2-RNAi lines and effect of this genetic manipulation in maize development is presented. BLAST searches showed that GSK3 family is represented with ten members in maize and phylogenetic analyses divided them into three clades. Five members clustered under “Clade II”, whose members have been shown to be involved in BR signaling in Arabidopsis. No maize members grouped with Arabidopsis Clade III. Suppression of BIN2 family by RNAi method resulted in plants with short stature. This was unexpected, because with the suppression of BIN2, a negative regulator of BR signaling pathway, increased BR signaling was expected to promote increased plant height. The short stature was due to shortened internodes at least partly caused by decreased cell elongation. Opposite to shorter plant height of bin2-RNAi plants, tassel internodes, leaf blades, and sheaths were longer compared to non-transgenic siblings. Contrary to bri1-RNAi lines, which had disrupted auricle morphology, bin2-RNAi lines tend to have larger auricles. These two opposite auricle phenotype suggest that BR signaling is central to control auricle development. One interesting phenotype of bin2-RNAi was
the changed kernel morphology when *bin2*-RNAi plants are used as female. Independent of their genotype, kernels on *bin2*-RNAi cobs were large, have pointy ends, and most of the time opaque. However, these traits were not seen when *bin2*-RNAi was used as pollen source. *bin2*-RNAi phenotypes were epistatic to *bril*-RNAi plants’ phenotypes when they crossed to each other, which is as expected given that BIN2 is located downstream of BR1I. This is supporting evidence that *bin2*-RNAi plants have increased BR signaling.

In summary, we manipulated two BR signaling genes in maize to understand the role of BR signaling in maize growth and development. There were exciting phenotypic characteristics in transgenic lines such as shorter stature and erect leaf habit which are desired agronomical traits. Manipulation of corn, an important crop, via these traits might increase its grain and biomass yield with the help of future studies. In conclusion, this dissertation is the first report on BR signaling in maize and it is a good start to understand the development and growth of maize via Brassinosteroid signaling.

References


Ye HX, Li L, Yin YH (2011) Recent Advances in the Regulation of Brassinosteroid Signaling and Biosynthesis Pathways. Journal of Integrative Plant Biology 53: 455-468

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The journey that took me the “Ph.D” degree was long, tiring, but worthy as well. Especially having this degree abroad in a different culture, as an international student was a little bit more challenging. I could not accomplish this without help from many people.

First of all, I would like to thank to my advisor Dr. Phil Becraft for his guidance, encouragement, and helpful discussions during these past years to accomplish what I have done. I always bothered him with many questions, but he answered and helped me always with patience. I also want to thank my Ph.D committee members: Drs. Erik Vollbrecht, Yanhai Yin, Maria Salas-Fernandez, and Michael Muszynski for their guidance.

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Last but not least, many thanks to my family and my wife for their support and prayers. I dedicate this thesis to my wife and parents.